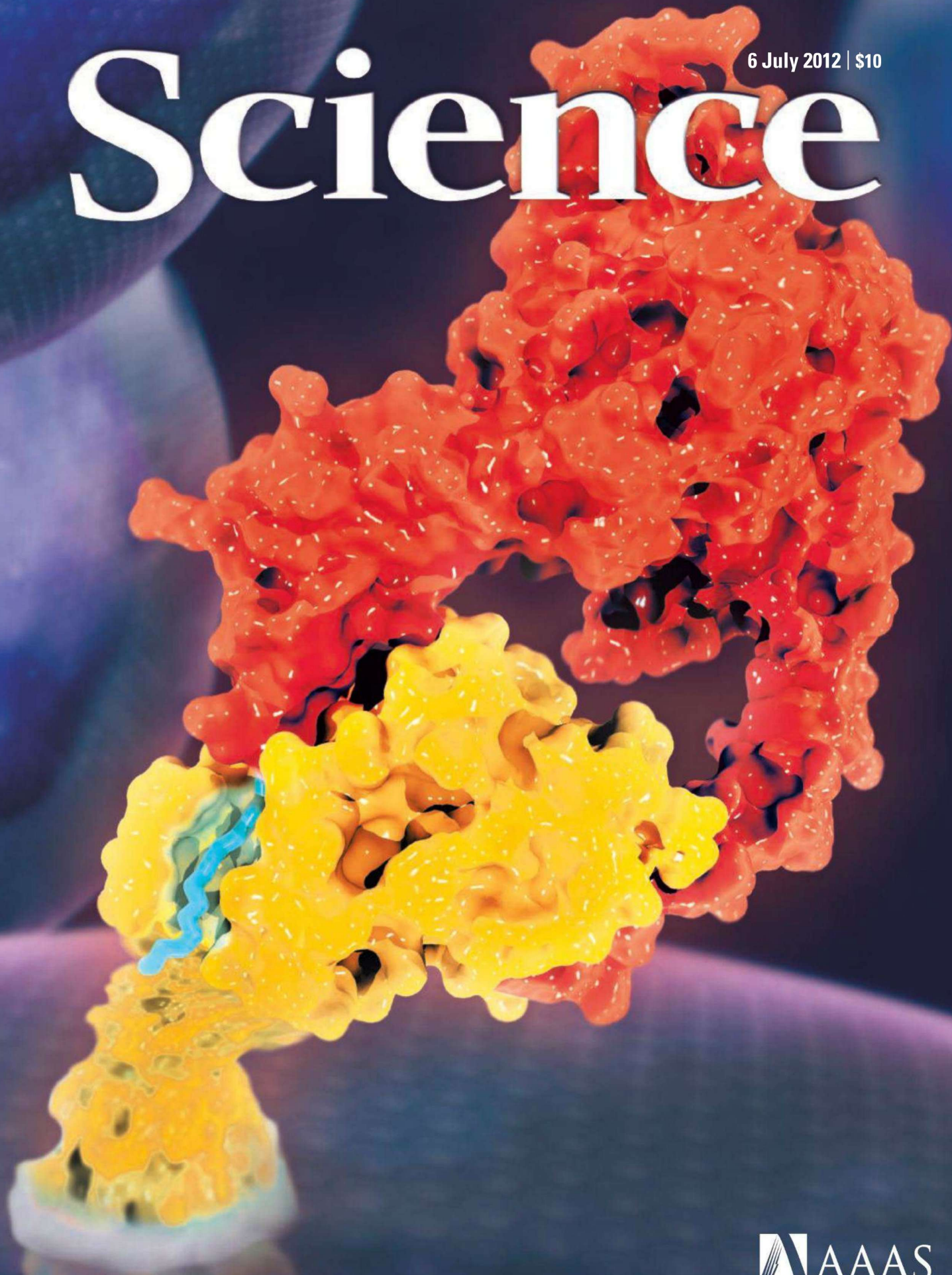


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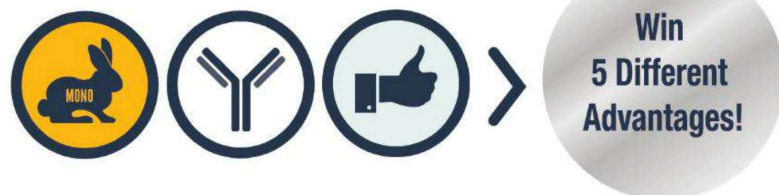
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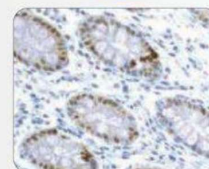
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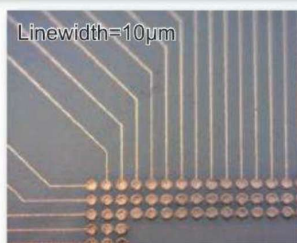
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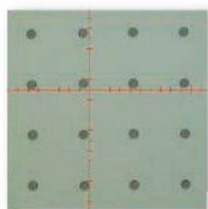
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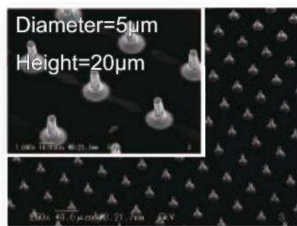
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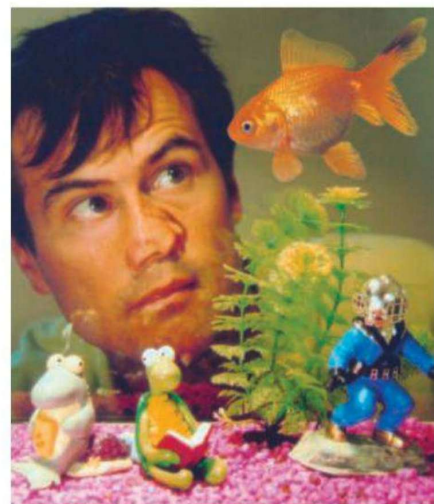
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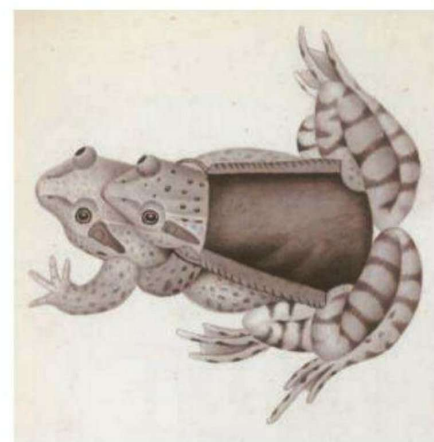
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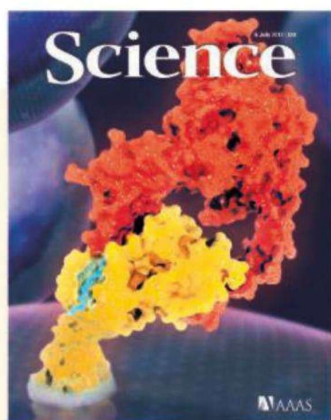
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COVER

Structure of the Wnt signaling molecule (red) in complex with the Frizzled ligand-binding domain (yellow), schematically depicted as connected to the cell surface. A key feature of this structure is the visualization of a lipid group (blue) on Wnt directly engaging Frizzled. The Wnt/Frizzled mode of binding paves the way for the design of Wnt-based therapeutics. See page 59.

Image produced by Eric Smith and Chris Garcia

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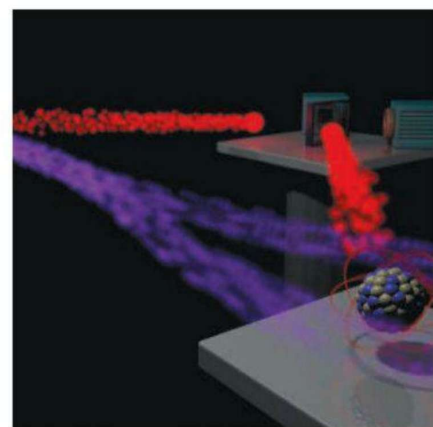
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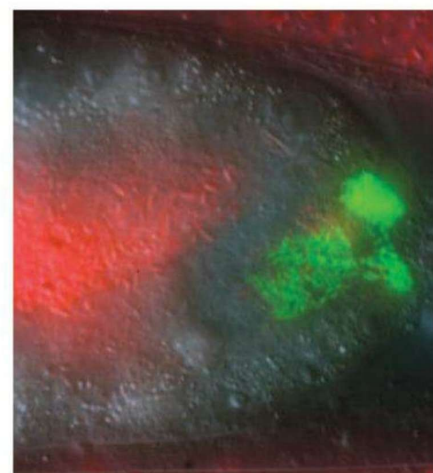
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Northeastern soils are recovering but still show signs of damage.

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Taken for Granted: A Stellar Opportunity

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Two reports propose significant reforms for graduate students and postdocs.

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Pushing Students Toward STEM

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<< A Long Collapse

Coral reefs are threatened by global warming and ocean acidification, and so it is important to understand better how and why environmental changes have affected them in the past. **Toth et al.** (p. 81) present a 6000-year-long record of coral reefs off the coast of Panama, Central America. The reefs effectively stopped growing for approximately 2600 years, beginning around 4000 years ago. This collapse of the coral reef system was probably caused by increased variability of ENSO, the El Niño–Southern Oscillation. If the strength or frequency of ENSO were to increase, the viability of these and other reef systems in the Pacific could be put further at risk.

Network, Network, Network

The fact that our interactions with others influence many of our decisions has led to research on characterizing the networks to which we belong and, more recently, on interventions that can change networks thereby changing behavior. This can have a variety of purposes, including promoting information flow through an organization or finding vulnerable points in bioterrorist networks. **Valente** (p. 49) reviews a variety of strategies for affecting networks.

Dissecting Wnt/Fz Interaction

Wnt proteins activate the transmembrane receptor Frizzled (Fz) to initiate pathways central to vertebrate and invertebrate development. Wnts are palmitoylated, which has complicated structural and functional characterization. **Janda et al.** (p. 59, published online 31 May; see the Perspective by **Bienz and He**) achieved coexpression and purification of *Xenopus* Wnt8 (XWnt8) with mouse Fz8 cysteine rich domain (CRD) and determined a crystal structure of the complex. Wnt binds to the Fz8 CRD at two distinct sites with the lipid group playing a key role in the first interface. Both interfaces involve conserved amino acids, which may explain the known pleiotropy of the Wnt/Fz interaction.

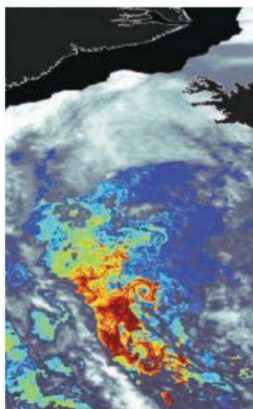
A Dwarf in the Making

It is a mystery how brown dwarfs, which have masses in between those of planets and stars,

form. Based on observations with the Institut de Radioastronomie Millimétrique Plateau de Bure Interferometer, located in the French Alps, **André et al.** (p. 69; see the Perspective by **Basu**) report the detection of a brown dwarf that is still in the process of forming. The results lend support to models that suggest that brown dwarfs form in the same way as nuclear-burning stars, like our Sun, but do not rule out other models.

Early Bloom Trigger

Springtime phytoplankton blooms occur when high nutrient concentrations are combined with abundant sunlight and a stratified upper ocean layer. It has been thought that stratification occurs because in the spring, seasonal warming causes the water to expand, making it less dense, which creates a layer resistant to mixing from below. Now, **Mahadevan et al.** (p. 54; see the Perspective by **Martin**) have combined observations of the upper water column from the subpolar North Atlantic with ocean model simulations, which demonstrate that the initial stratification can be triggered by the dynamic effects of passing ocean eddies. These eddies can advance the time of the bloom by 20 to 30 days.



Entanglement On Cue

Quantum entanglement between particles lies at the core of quantum mechanics. For practical applications such as long-distance quantum communications, cryptography, and quantum networks, entanglement must be heralded. That is, a signal flags the existence of the entangled state so that it can be manipulated and transferred. **Hofmann et al.** (p. 72; see the Perspective by **Volz and Rauschenbeutel**) excited trapped single atoms in different laboratory rooms 20 meters apart and were able to show that manipulation, interference, and detection of the photons from the excited atoms could signal when the atoms are entangled. The extent of the observed entanglement may be sufficient to start to probe fundamental questions relating to the entanglement process and thus address the foundations of quantum mechanics.

Mr. Cool

Laser cooling of atoms relies on the presence of electronic transitions of specific structure, making it practical for a limited number of atomic species at relatively low densities. Alternative methods include those involving optical cavities, where atom-cavity interaction enables cooling without the need for resonant transitions. **Wolke et al.** (p. 75) present such a cooling scheme that was used to heat and then cool a Bose-Einstein condensate of Rb atoms. The method should be applicable to hotter samples where a sequence of laser pulses of different frequencies would need to be used.

Indirect Injection

Aerosols in the stratosphere, especially sub-micron-hydrated sulfuric acid droplets, are an important factor influencing climate variability. Stratospheric sulfate aerosols can form from sulfur dioxide that has been transported from the underlying troposphere. Large volcanic eruptions can inject sulfur dioxide and other material into the stratosphere, but smaller volcanoes have been thought not to be energetic enough to do so. **Bourassa et al.** (p. 78) used satellite data to show that sulfur dioxide from the 2011 eruption of the Nabro stratovolcano in Eritrea was lofted into the stratosphere by deep convection associated with the Asian summer monsoon.

Twin Tales of Two Toxins

The luminescent bacterium, *Photorhabdus luminescens*, is carried in the gut of an insect-parasitic nematode as a stealth weapon. By using

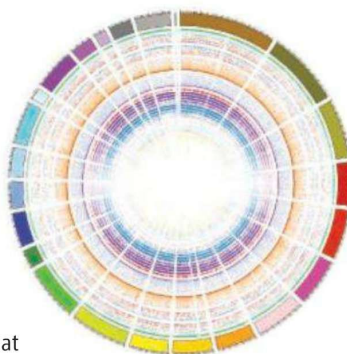
an allele swapping technique, **Somvanshi *et al.*** (p. 88) investigated the promoter-switching mechanism that flips the bacterium from the almost dormant M forms, which stick to the adult nematode's posterior gut, into the motile, luminous P forms, which are armed with the toxic virulence factors needed to overcome the insect prey of the nematode. Similar switches may operate in bacteria that flip between harmless commensals and lethal pathogens. The bio-control agent *Bacillus thuringiensis* also kills insects by means of a crystal toxin, which allows the bacteria to penetrate the host gut and access nutrients. Release of nutrients also allows bacterial cheats that do not make toxin, to grow and outcompete the toxin-producing colonizers. In field experiments, **Raymond *et al.*** (p. 85) found that, consequently, the bacterial population becomes less virulent. Because these type of virulence factors are secreted from the cell and are widespread in pathogens, such social interactions may affect the fitness and constrain the virulence of many toxin-producing bacteria.

Letting Pyruvate In

Transport of pyruvate is an important event in metabolism whereby the pyruvate formed in glycolysis is transported into mitochondria to feed into the tricarboxylic acid cycle (see the Perspective by **Murphy and Divakaruni**). Two groups have now identified proteins that are components of the mitochondrial pyruvate transporter. **Bricker *et al.*** (p. 96, published online 24 May) found that the proteins mitochondrial pyruvate carrier 1 and 2 (MPC1 and MPC2) are required for full pyruvate transport in yeast and *Drosophila* cells and that humans with mutations in MPC1 have metabolic defects consistent with loss of the transporter. **Herzig *et al.*** (p. 93, published online 24 May) identified the same proteins as components of the carrier in yeast. Furthermore, expression of the mouse proteins in bacteria conferred increased transport of pyruvate into bacterial cells.

A Deep Look Into Our Genes

Recent debates have focused on the degree of genetic variation and its impact upon health at the genomic level in humans (see the Perspective by **Casals and Bertranpetit**). **Tennessen *et al.*** (p. 64, published online 17 May), looking at all of the protein-coding genes in the human genome, and **Nelson *et al.*** (p. 100, published online 17 May), looking at genes that encode drug targets, address this question through deep sequencing efforts on samples from multiple individuals. The findings suggest that most human variation is rare, not shared between populations, and that rare variants are likely to play a role in human health.



Cancer Gene Islands

Human tumors are riddled with genomic alterations that rearrange, remove, amplify, or otherwise disrupt a wide spectrum of genes, and a key challenge is identifying which of these alterations are causally involved in tumorigenesis. The role of recurrent hemizygous focal deletions is especially puzzling because these deletions preferentially affect certain chromosomal regions and result in the loss of one copy of a whole cluster of adjacent genes. **Solimini *et al.*** (p. 104, published online 24 May; see the Perspective by **Greenman**) found that these deletions span genomic regions that are enriched in genes that negatively regulate cell proliferation. The cumulative reduction in dosage and tumor suppressive function of the genes within these "cancer gene islands" may represent a critical factor driving tumor growth.

You Must Be Human

Are there specific brain structures associated with social cognition or with aspects of information processing that frequently occur together with social cognition? **Carter *et al.*** (p. 109) invited subjects to play a simplified virtual poker game against either a human or a computer and examined brain scans collected during the game. The brains scans when the cards shown were used to predict the participant's decision 6 seconds later. Activity in one region, the temporal-parietal junction, was one of the best predictors of future decisions against a human opponent, but the single worst predictor against a computer opponent.

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The Internet and Academic Freedom

THE INTERNET IS AS VALUABLE A RESEARCH TOOL AS ANY MICROSCOPE OR MAGNETIC RESONANCE imaging scanner. It was shaped by researchers and academics who wanted to collaborate across campuses, then across borders. Many aspects of scholarship are now conducted online. This is particularly true in the sciences. Researchers around the globe store data in the “cloud” and analyze those data with software that requires connectivity. And critical discussions are routinely conducted by e-mail and videoconferencing. But the academic freedom that is often taken for granted depends on an Internet that is maintained as an open platform for the free exchange of information and ideas—or “Internet freedom.” At present, Internet freedom is threatened across the globe, and this should concern scientists and other academics as much as it does human rights activists.

At the same time that online information and education are vastly increasing access to knowledge and greatly improving opportunities for individuals and institutions in developing nations, more governments are seeking to control their citizens’ peaceful online activities by blocking or filtering content. This interference takes endlessly inventive and sometimes nefarious forms, including the use of advanced surveillance technologies and spyware that leads to human rights abuses. In a number of cases, governments have shut down the Internet entirely as a means of political control. According to the OpenNet Initiative, 47% of the Internet’s users live in countries that block legitimate content.* The blocked content isn’t only material judged to be political. It can include scientific topics considered controversial, such as evolution.†

As universities and laboratories become more and more digitized, these restrictions on the Internet will increasingly impinge on academic life and on the opportunities for global academic collaboration. One disturbing recent example is the Iranian government’s crackdown on the Baha’i Institute for Higher Education, an online university serving members of the Baha’i faith.‡ In response to a government rule barring those of the Baha’i faith from postsecondary education, the institute had founded an online correspondence school. Last year, seven professors and officials involved with the institute were sentenced to a total of 30 years’ imprisonment.

Within the past several months alone, the instances of Internet harassment and illegitimate surveillance of academics and students include the following: Student communications have been monitored and controversial conversations ended, hundreds of students have been expelled for what they posted on social media, and students and professors have been arrested for the expression of peaceful views.§ Last year, the governments of China and Russia, with support from others, came to the United Nations to suggest the need for an International Code of Conduct for Information Security. Were such a code to be adopted, it would inevitably erode online freedoms by legitimizing content control. This is only one of several efforts by governments to use international frameworks and institutions to control Internet content. Such official challenges to open use of the Internet are likely to grow more intense in the years ahead.

The Internet cannot be maintained as an open and global network unless government, business, and civil society work together. The U.S. State Department, with a coalition of like-minded countries, has made protecting global Internet freedom a foreign policy priority (see www.humanrights.gov/ifreedom). The scientific community, which benefits so dramatically from an open Internet, must become a leader in this effort by uniting with colleagues in their universities and scientific societies to promote Internet freedom. People everywhere have the right to participate freely online. We urge the academic community to remain in the vanguard of one of the seminal free speech issues of our time.

— Michael H. Posner

10.1126/science.1226099



*opennet.net/blog/2012/04/global-internet-filtering-2012-glance. †news.sciencemag.org/scienceinsider/2011/12/controversial-turkish-internet-c.html. ‡www.iranhumanrights.org/2011/05/bahai-university-attacked/. §Read about these cases and others at concernedscientists.org and scholarsatrisk.nyu.edu.



OCEAN SCIENCE
Going Up

Models of dynamic sea-level rise have demonstrated that sea-level increases caused by global warming will not be the same in all locations, because changes in ocean circulation, temperature, and salinity; the redistribution of mass owing to the melting of ice sheets; and the resulting changes in the shape and rotation of Earth all affect the regional expression of sea surface height. These models predict that one region in which sea level should rise most rapidly is along the northeast coast of the United States. Sallenger *et al.* examined tide gauge records from around the entire perimeter of the county, finding that such a hot spot already exists and that the rate at which sea level is rising there is three to four times the global average, consistent with model predictions. The rise is also correlated to the rate of melting of the Greenland ice sheet, in concert with a slowing of deep circulation in the Atlantic. — HJS

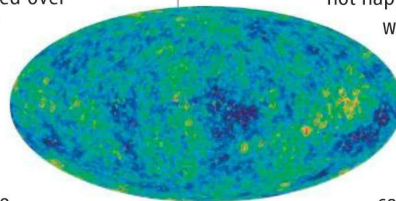
Nat. Clim. Change **2**, 10.1038/NCLIMATE1597 (2012).

ECOLOGY

Don't Discount the Deep

The deep sea is one of the least-known regions on Earth. Its inaccessibility, however, should not be equated with a lack of biological relevance. Deep-sea corals, often referred to as cold-water corals (CWCs), are a case in point. Tropical coral habitats are known for their diversity, and they provide essential ecosystem functions for many marine taxa. As a result, they receive substantial conservation consideration. It has been suggested that CWC systems are also important biodiversity hot spots and may serve as essential fish nurseries, but without direct evidence, their

protection is minimal. Baillon *et al.* searched marine research trawls conducted over 5 years off the eastern coast of Canada for evidence of a fish nursery role for CWCs. They found fish embryos associated with five species of sea pens and used genetic data to identify them as belonging to several species, including two species of redfish (*Sebastes* spp.), which are commercially important and listed as either endangered or threatened in Canada. Although much remains to be learned about CWC systems, these results confirm that they act as fish nurseries. Fur-



thermore, they suggest that like tropical coral systems, CWCs deserve serious conservation and policy consideration. — SNV

Front. Ecol. Environ. **10**, 10.1890/120022 (2012).

CELL SIGNALING

Keeping a Lid on Signaling

Keeping growth factor receptors turned off when appropriate is just as important as proper activation by ligand binding. This is demonstrated by the developmental abnormalities and cancer-promoting effects ascribed to the uncontrolled activity of such receptors. Lin *et al.* propose a mechanism by which cells may be protected from unwanted phosphorylation of targets by the fibroblast growth factor receptor (FGFR), a receptor tyrosine kinase. The adaptor protein Grb2 is normally thought to promote receptor signaling by binding to phosphorylated residues on the receptors and interacting with other signaling proteins. In cultured human cells transfected with FGFR, however, Grb2 associated with unstimulated FGFR and was required for a basal amount of autophosphorylation of the receptor. Such basal phosphorylation appeared not to activate signaling, however, because the bound Grb2 sterically inhibited further receptor autophosphorylation. The authors propose that when the receptor becomes stimulated by its ligand, Grb2 itself is phosphorylated by the receptor, resulting in its release and relief of its inhibitory effect on receptor signaling. — LBR

Cell **149**, 1514 (2012).

ASTROPHYSICS

Textures in the Sky?

Theories of high-energy physics predict that topological defects formed in the early universe as a result of symmetry-breaking phase transitions. As the universe cooled and expanded, it underwent phase changes in which the different forces decoupled and the symmetries between elementary particles broke. These transitions did

not happen in the same way everywhere in the universe, and as misalignments in the arrangement of atoms lead to defects in crystals, misalignments in symmetry breaking led to cosmic defects. Textures are

one of the types of defects predicted by theory and should generate characteristic hot and cold spots in the oldest image we have of the universe—the cosmic microwave background, emitted when the first atoms formed 13.7 billion years ago. To test these predictions, Feeney *et*

al. used state-of-the-art Bayesian methods to analyze full-sky microwave background data from the Wilkinson Microwave Anisotropy Probe. They show that these data are consistent with the absence of cosmic textures and rule out any theories that predict more than six detectable textures on the full sky. Data from the Planck satellite may soon provide better constraints. If detected, these features would probe physics at energies much higher than those that can be reached at the Large Hadron Collider. — MJC

Phys. Rev. Lett. **108**, 241301 (2012).

CHEMISTRY

Four Different Corners

Photochemical dimerization of olefins can yield a wide variety of symmetrically substituted cyclobutane derivatives. The bigger challenge is generating a carbon square with a different substituent at each corner. Two different olefins can pair up with each other in multiple distinct combinations and orientations, risking a messy mixture. Yet nature appeared to have overcome the obstacles in a class of compounds isolated recently from the plants that produce black pepper. Two studies demonstrate complementary synthetic routes to these tetrasubstituted cyclobutanes. Gutekunst *et al.* used photochemistry at the outset, but in an intramolecular context, to bias the relative olefin orientations and set two distinct opposing corners. This left unsubstituted CH₂ centers at the remaining corners, and the authors applied direct palladium-catalyzed vinyl and aryl iodide additions to establish the basic skeleton before functional elaborations. Liu *et al.* skirted photochemistry altogether, setting three corners by expanding a triangular cyclopropane precursor outward to a pendant carbene center. This yielded a cyclobutene poised for rhodium-catalyzed conjugate addition of an arylboronic acid to set the final corner. Close comparison of these products to the natural isolate revealed that the pepper plant was actually synthesizing an isomer with a six-membered (rather than four-membered) ring. — JSY

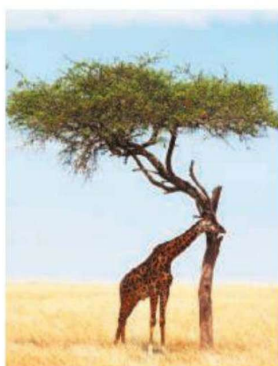
Angew. Chem. Int. Ed. **51**, 10.1002/anie.201203897; 10.1002/anie.201203379 (2012).

ECOLOGY

Drivers of Diversity

Large-scale biogeographic patterns of species diversity are generally attributed to the effects of regional climate, climatic history, and other physical factors such as topography. Greve *et al.* now report a case where continental-scale diversity patterns appear to also be influenced by biotic factors. In Africa, the diversity of acacia

trees reaches its highest levels in East Africa and the Limpopo Basin, and the species richness of browsing herbivores (herbivores that use acacias as a food source) is the best explanatory driver of this pattern. The mechanism behind this association remains uncertain; however, one possibility is



that decreased fitness and decreased competition of trees under heavier pressure from browsers could lead to greater opportunities for the coexistence of multiple tree species. If consumer diversity has indeed

contributed to acacia diversity, the conservation and retention of large herbivores in the African landscape may ultimately be important to the persistence of a diverse tree community. — AMS

J. Ecol. **100**, 10.1111/j.1365-2745.2012.01994.x (2012).

MOLECULAR BIOLOGY

Mighty Miniscule RNAs

An ever-increasing number of small RNAs—ranging in size from ~20 to 400 nucleotides (nt) in length—are being found that regulate gene expression in both prokaryotes and eukaryotes. A class of tiny RNAs, known as nanoRNAs because of their minuscule size (~2 to 4 nt), have previously been shown to prime transcription initiation *in vitro* by being directly incorporated into a target transcript.

By overexpressing the “nanoRNases” oligoribonuclease (Orn) and RnB, Vvedenskaya *et al.* were able to reduce the levels of nanoRNAs in *Escherichia coli* to demonstrate that for specific genes, the tiny RNAs can influence transcription start-site selection during stationary phase growth. The nanoRNAs were also able to regulate some of these genes (*tomB* and *bhsA*), stimulating their levels during stationary-phase growth. Genome-wide analysis indicated that the promoters of many of the genes sensitive to growth phase-dependent nanoRNA-mediated start-site control have a specific sequence element that determines, in part, whether the promoter will be targeted by nanoRNA-mediated priming. NanoRNAs might stimulate *de novo* transcription initiation, or they could increase RNA stability through the addition of a U and/or a hydroxyl (both characteristic of nanoRNAs) to the 5' end of the RNA. — GR

Gene. Dev. **26**, 10.1101/gad.192732.112 (2012).

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Meinrat O. Andreae, Max Planck Inst., Mainz
Johan Auwerx, EPFL
David Awschalom, Univ. of California Santa Barbara
Ben Barres, Stanford Univ.
Jordi Bascompte, Estación Biológica de Doñana, CSIC
Raymond Batista, London Research Inst.
Fay H. Baughman, Univ. of Texas, Dallas
David Baum, Univ. of Wisconsin
Mark Bear, Massachusetts Inst. of Technology
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Ian Boyd, Univ. of St. Andrews
Christian Büchel, Universitätsklinikum Hamburg-Eppendorf
Joseph A. Burns, Cornell Univ.
William P. Butz, Population Reference Bureau
György Buzsáki, Rutgers Univ.
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James Collins, Boston Univ.
Alan Cowman, Walter & Eliza Hall Inst.
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Wolfgang Cramer, Medit. Inst. for Ecology & Paleocology
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Tom Daniel, Univ. of Washington

Frans de Waal, Emory Univ.
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Barry Everitt, Univ. of Cambridge
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LIFE SCIENCE TECHNOLOGIES

Nanotechnology

Prognosis for Nanotechnology Treatments

In This Issue

Nanotechnology-based tools and treatments promise sensitive disease diagnosis and accurate drug delivery. Most important, the physical features of nanometer-size materials provide capabilities that larger objects cannot match. Nonetheless, some of the challenges, such as biocompatibility and toxicity, require ongoing research and improved solutions. Despite these obstacles, some nanoscale approaches to medicine can already be used.

See full story on page 112.

Upcoming Features

Proteomics: Clinical Diagnostics—August 31

Genomics: Epigenomics—October 26

Tissue Engineering: 3-D/Scaffolding—December 7

P-1000

Next
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Micropipette
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The latest in micropipette pulling technology!

FEATURES

- Color touch-screen interface
- Safe heat mode to protect and extend filament life
- Pipette Cookbook program directory
- Line repeat mode simplifies programming
- Glossary with micropipette and puller terminology
- Copy & Paste function for writing new programs
- Two symmetrical pipettes with each pull
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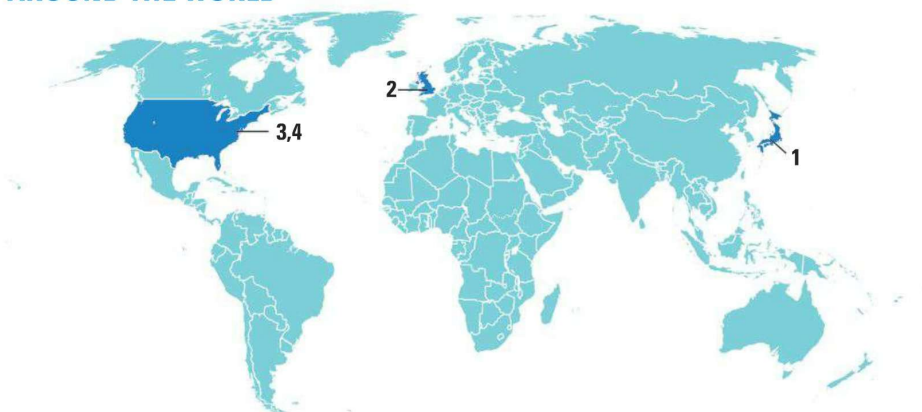
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AROUND THE WORLD



Ohi, Japan 1

Japan Reboots Nuclear Power, But Eyes Renewables

Japan's Kansai Electric Power Company powered up one of their nuclear reactors at its Ohi plant on 1 July, making it the first reactor to restart since the 2011 Fukushima Daiichi nuclear crisis.

Scientists questioned the wisdom of the decision. For instance, faults crossing the site could produce stronger-than-anticipated shaking during an earthquake, warns seismologist Katsuhiko Ishibashi, professor emeritus at Kobe University in Japan.

Reactors must be shut down periodically

for maintenance, and by custom, local governments used to approve each restart. After the March 2011 tsunami led to massive radiation leaks from the Fukushima Daiichi plant, local opposition kept the shut reactors offline. But facing summer energy shortages, the national government stepped in

and approved restarting two Ohi reactors.

Last week, the national government also announced that it would heavily subsidize solar, wind, and geothermal power to reduce the country's reliance on nuclear power. Previously, considering renewables was practically impossible, says alternative energy advocate Tetsunari Iida. The Fukushima disaster "drastically changed Japan's nuclear energy policy," he says.



London 2

New Science Adviser for the U.K.

The director of the Wellcome Trust, the world's wealthiest funder of biomedical research, has been appointed as the U.K. government's next chief scientific adviser. Mark Walport will replace John Beddington, the population biologist who has held the post since 2008, next April.

Walport, a physician specializing in immunology who was a professor of medicine at Imperial College London before coming to Wellcome, will assume the role at a challenging time, with the U.K. science budget frozen for several years.

University of Oxford neuroscientist Colin Blakemore, former head of the U.K. Medical Research Council, welcomes the appointment, saying in a statement: "Mark Walport is bright, efficient, and enormously knowledgeable about science, education, and innovation. But, equally important, he has great political acumen and robust independence. I can't think of anyone better prepared to make the case for the use of science in government and for the defence of the best of British science."

<http://scim.ag/sci-adv>



Walport

Washington, D.C. 3

Supreme Court Upholds Health-Care Reforms

There were surprises in the U.S. Supreme Court's ruling last week that upheld the constitutionality of the health care reform bill: The decision was far narrower than the preliminary hoopla suggested it might



be, and it was secured with the help of a Republican appointee to the court, Chief Justice John Roberts. He joined four liberal-leaning justices to validate the so-called "individual mandate" requiring every citizen to have or purchase health insurance as a tax. But the court rejected the argument that the mandate was equally justified as a federal regulation of commerce. Conservatives welcomed that part of the decision and said they would try to get Congress to repeal the Patient Protection and Affordable Care Act (PPACA).

Health-care organizations for the most part expressed relief that the legality of PPACA was affirmed. And some PPACA-linked health programs that could have been scrapped—including the authority to regulate "biosimilar" drugs, a fund for preventive health work, and a fund for comparative effectiveness treatment research—survived intact. PPACA's scheme for funding Medicaid programs was rejected, however, and will have to be reconfigured.

Washington, D.C. 4

Climate Science Gets a Hug In U.S. Court Decision

A U.S. federal appeals court on 26 June ruled that the Environmental Protection Agency (EPA) relied on sound science in deciding that greenhouse gases potentially "endangered" public health and welfare.

In a unanimous decision, a three-judge

NOTED

>The United Kingdom's Wellcome Trust is adding teeth to a requirement that **grantees post their papers in a public database** within 6 months of publication in a journal. Only 55% of eligible papers are now being deposited. To boost that rate, the Wellcome Trust will withhold final grant payments and new awards and renewals to researchers who fail to comply.

panel rejected the states' and industry groups' arguments that EPA had improperly "delegated" its scientific judgment on greenhouse gases by relying on climate change assessments developed by the Inter-governmental Panel on Climate Change, the U.S. Global Change Research Program, and the National Research Council of the U.S. National Academies.

"EPA simply ... sought out and reviewed existing scientific evidence to determine whether a particular finding was warranted," the judges wrote.

The ruling has its roots in a 2007 U.S. Supreme Court decision stating that EPA could regulate carbon dioxide and other greenhouse gases under the Clean Air Act if the agency demonstrated that they threatened public health. In 2009, EPA issued rules limiting emissions from power plants, factories, and vehicles, which drew dozens of legal challenges that were consolidated into the current case. http://scim.ag/_epa

NEWSMAKERS

Three Q's

Cornell University is developing a slick new tech campus in New York City. Last week, it announced its first high-profile faculty hire:



Estrin

University of California, Los Angeles's (UCLA's) **Deborah Estrin**, a computer scientist known for her work in embedded network sensing and frequent presence at the top of tech leader lists.

Q: Why are you joining CornellNYC Tech?

Because of the incredible alignment of the conception of [CornellNYC Tech] with the work I do and how I like to do it. The campus is all about embedding research and education with the concept of its application, and the way I like to do my work is with very tight co-innovation with the application. When I read the proposal, it was just something I couldn't resist.

Q: Why leave an established university like UCLA?

It's the draw of joining something that's so like a start-up. [CornellNYC Tech] is connected to a great university but the whole concept of the campus is in line with innovation and entrepreneurship. It's not secondary to how it does its research.

Easy Does It

A one-of-a-kind tree has been spared the ax at the Fairchild Tropical Botanic Garden in Miami, Florida. *Haldina cordifolia* is native to China, India, and the Malaysian Peninsula, where it is harvested for its timber and its bark is used as an antiseptic. In 1937, a U.S. Department of Agriculture researcher named Walter Koelz collected a seedling in India; in 1940, it was planted at the then 2-year-old botanic garden. Today, the 23-meter-tall specimen is the only *Haldina* growing in the United States. When the botanic garden needed space to build a new building, they decided to move the tree rather than lose it. Arborists spent a year gradually pruning its roots. Then in June, two cranes hoisted the 12,700-kilogram tree about 30 meters to a new location. Nannette Zapata, the garden's chief operating officer, says so far the tree is doing fine.



Q: What are you working on at the moment?

My primary focus is mobile health (using mobile devices such as cell phones to collect health data). We look at innovative forms of data capture and use ... to help tailor clinical interventions, particularly around chronic illnesses like depression, diabetes, and gastrointestinal illnesses, where the burden of care and understanding is on the individual.

'Best Doughnut' Discoverer Wins Ramanujan Prize

Fernando Codá Marques, a differential geometer at the National Institute for Pure and Applied Mathematics (IMPA) in Rio de Janeiro, on 28 June was named the winner of the 2012 Ramanujan Prize for Young Mathematicians from Developing Countries.

In 2009, Marques used methods pioneered by Grigori Perelman (whose proof of the Poincaré conjecture was *Science's* Breakthrough of the Year in 2006) to show that any two positively curved spaces with identical topologies can be gradually deformed into one another without losing the positive cur-



Marques

THEY SAID IT

"EPA is not required to re-prove the existence of the atom every time it approaches a scientific question."

—The U.S. Court of Appeals for the District of Columbia Circuit in an opinion upholding the U.S. Environmental Protection Agency's power to regulate greenhouse gases as pollutants (*Science*, 6 July, p. 18).

vature property. The result provides a useful tool for cosmologists studying the shape of the universe. This year, Marques and Andre Neves of Imperial College London proved the 47-year-old Willmore conjecture on the minimal bending energy for a torus. They showed that the optimal doughnut has a hole that is 17% as wide as the doughnut itself, and that its bending energy is lower than that of any cruller.

"Fernando is also a fantastic lecturer and a wonderful person to discuss mathematics with," says mathematician Robert Kusner of the University of Massachusetts, Amherst. "I can't think of a more deserving recipient of the Ramanujan Prize."

Random Sample

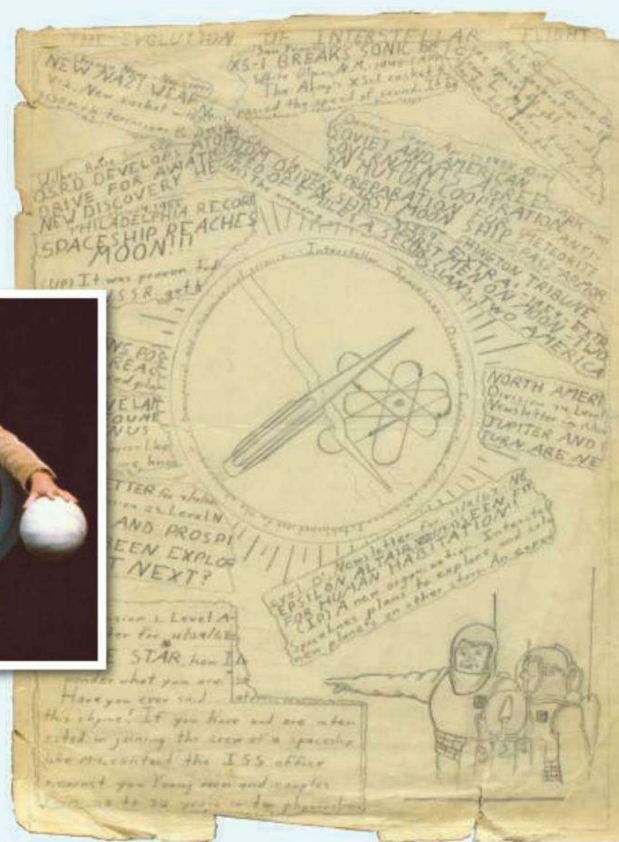
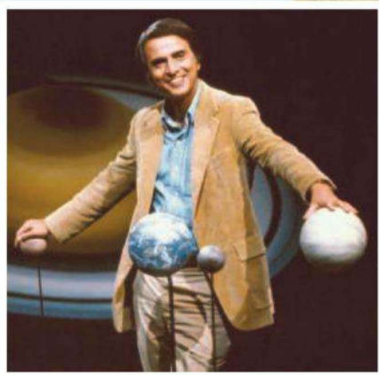
Piecing Together Carl Sagan

Archivists at the Library of Congress in Washington, D.C., face a daunting task: painting a picture of noted astronomer Carl Sagan using 750,000 objects and documents he saved over the course of his life.

The Seth MacFarlane Collection of the Carl Sagan and Ann Druyan Archive, donated to the library by television producer and Sagan fan Seth MacFarlane, contains notes, doodles, and correspondence from the astronomer who brought the universe into people's living rooms. The sheer number and diversity of items will keep the archive team busy for a full year.

This is a multimedia collection, says Leonard Bruno, project head and science manuscript historian at the library. "There are lots of photos, videotapes, audio cassettes, technical reports from his work with NASA, a quilt with mathematical equations on it, and even a dry erase board with the story board for Sagan's movie, *Contact*, Bruno says. The collection also includes the scientist's report cards, undergraduate notes, fan mail, and early research. Archivists must prepare each article separately, carefully examining, grouping, and placing them in storage containers that will protect them from the environment.

This new collection takes its place alongside scientific manuscripts from figures such as Thomas Edison, Alexander Graham Bell, and E. O. Wilson. The library plans to open Sagan's works to the public in November 2013.



>>FINDINGS

What Gets Yaks High?

Nomads on the Tibetan plateau have relied on yaks instead of cows as their beasts of burden for the past 4000 years. Although the two are closely related—having diverged only 4.9 million years ago, around the same



time that humans and chimpanzees parted ways—the domestic yak (*Bos grunniens*) is superbly adapted to the region's extreme elevations, which can exceed 4500 meters. Now researchers have uncovered the genet-

ics behind this ability. The genome of a female domestic yak, reported on 1 July in *Nature*, reveals several genes that make it more suited for heights. Three genes help the animal regulate its body's response to hypoxia, or oxygen deprivation, at high-altitudes, and five genes help it optimize the energy it gets from its food, which is scarce on the plateau. Cattle lack these genes, which is why they cannot survive at the same heights as their bovine cousins. By understanding which genes are needed to live successfully at high elevations, the researchers say, scientists may be able to better treat and prevent altitude sickness and hypoxia-related complications such as high-altitude cerebral edema and high-altitude pulmonary edema, which can be fatal in humans.
<http://scim.ag/yak-gene>

Science LIVE

Join us on Thursday, 12 July at 3 PM EST for a live chat with experts on the Higgs boson.
<http://scim.ag/science-live>

BY THE NUMBERS

60,000 Number of signatures gathered by the Libel Reform Campaign on a petition to amend U.K. libel laws to protect scientists, science writers, and others defending the "public interest."

76% Percentage of patents from 10 patent-prolific U.S. universities with at least one foreign-born inventor, according to a 26 June study from the Partnership for a New American Economy.

5 million Number of babies born via fertility techniques, including in vitro fertilization, since the first "test tube" baby was born in 1978, according to the nonprofit International Committee Monitoring Assisted Reproductive Technologies.

SCIENTIFIC ETHICS

Fraud-Detection Tool Could Shake Up Psychology

AMSTERDAM—The most startling thing about the latest scandal to hit social psychology isn't the alleged violation of scientific ethics itself, scientists say, or the fact that it happened in the Netherlands, the home of fallen research star and serial fraudster Diederik Stapel, whose case shook the field to its core less than a year ago. Instead, what fascinates them most is how the new case, which led to the resignation of psychologist Dirk Smeesters of Erasmus University Rotterdam and the requested retraction of two of his papers by his school, came to light: through an unpublished statistical method to detect data fraud.

The technique was developed by Uri Simonsohn, a social psychologist at the Wharton School of the University of Pennsylvania, who tells *Science* that he has also notified a U.S. university of a psychology paper his method flagged. That paper's main author, too, has been investigated and has resigned, he says. As *Science* went to press, Simonsohn said he planned to reveal details about his method, and both cases, as early as this week.

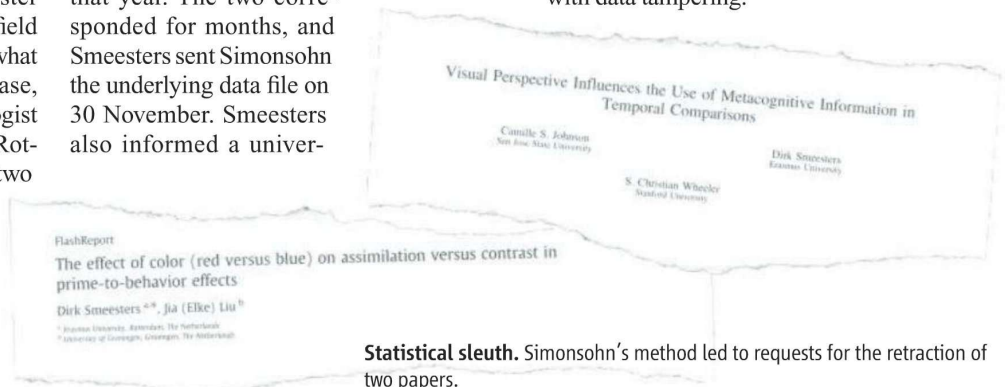
If it proves valid, Simonsohn's technique might find other possible cases of misconduct lurking in the vast body of scientific literature. "There's a lot of interest in this," says Brian Nosek of the University of Virginia in Charlottesville, who recently launched an examination of replicability in social psychology findings (*Science*, 30 March, p. 1558).

The method may help the field of psychological science clean up its act and restore its credibility, he adds—but it may also turn colleagues into adversaries and destroy careers. The field will need ample debate on how to use it, Nosek says, much the way physicists had to grapple with the advent of nuclear physics. "This is psychology's atomic bomb," he says.

Simonsohn already created a stir last year with a paper in *Psychological Science* showing that it's "unacceptably easy" to prove almost anything using common ways to mas-

sage data and suggesting that a large proportion of papers in the field may be false positives. He first contacted Smeesters on 29 August 2011 about a paper on the psychological effects of color, published earlier that year. The two corresponded for months, and Smeesters sent Simonsohn the underlying data file on 30 November. Smeesters also informed a univer-

on 21 June, 4 days before it released the investigation commission's report.) Smeesters says he can't judge Simonsohn's method because he's not a statistician. But he says that the odd data patterns found by Simonsohn emerged because of what he calls "questionable research practices" that aren't uncommon in his field, such as doing multiple analyses and picking the most convincing one, or leaving out certain subjects. Simonsohn says that explanation doesn't add up: "The raw data he sent me is not consistent with dropping observations selectively, but they are consistent with data tampering."



Statistical sleuth. Simonsohn's method led to requests for the retraction of two papers.

sity official about the exchange. Simonsohn says he was then contacted by the university.

An investigative commission set up in January by Erasmus reviewed Simonsohn's unpublished method with the help of two outside statisticians and concluded it was valid. When the panel subjected the rest of Smeesters's work to the same method, it found two more papers—one of them unpublished—that failed the test. Digging further, the commission reports that it discovered other problems; for instance, Smeesters said he no longer had the raw data for the three papers due to a computer crash at his home in September—

which he had mentioned on Facebook—while versions of the data recorded on paper were lost when he moved office. In a report released on 25 June, the commission said it couldn't prove that Smeesters had committed fraud, but it doubted the credibility of his explanations for the loss of data, and it had "no confidence in the scientific integrity" of the three papers.

Smeesters, who insists he did not make up data, tells *Science* that he stepped down in January for medical reasons, including feeling burned out and having a bad knee. (The university officially accepted his resignation

News of the affair had psychologists and statisticians yearning for details of Simonsohn's method. Initially, his name and details of his work were redacted from the released university report because his study remains unpublished. Three days later, with Simonsohn's permission, the university released an unredacted version, which offers more clues. According to the report, Simonsohn's method rests on the assumption that if a study measures the same variable in groups that supposedly are independent samples from the same population, chance will produce a certain variance between the means of those groups; if a paper reports means that have too little variance, that may be a sign that the data have been manipulated. A simulation can reveal the probability that this has happened in a given study.

Simonsohn says it would have been unethical for him to release his paper—and accuse Smeesters and the U.S. scientist—before the universities had finished investigating. The full paper and supplemental materials will clarify the method, he says.

"Uri will be vindicated when more details are made public," Phil Fernbach, a marketing researcher at the University of Colorado, Boulder, predicted in a comment on *Science*'s website last week. "He is a careful scientist who would never make such seri-

"We need to be sure that this tool is used properly, fairly, and wisely."

—DANIEL GILBERT,
HARVARD UNIVERSITY

ous accusations if the evidence weren't overwhelming."

Based on details in the university report, Richard Gill, a statistician at Leiden University in the Netherlands, carried out some data simulations following what he believes is Simonsohn's procedure. On his own blog, Gill concluded that "Simonsohn has probably found a useful investigative tool," but he criticized details of the way the university commission had applied it to Smeesters's work; he said it was akin to a "medieval instrument of torture: the accused is forced to confess by being subjected to an onslaught of vicious p-values which he does not understand."

Simonsohn says he has not read the analysis but objects to Gill's language. "If it wasn't targeted towards people trying to reduce fraud in science, the sophomoric tone would be amusing," he says. Geurt Jongbloed, a statistician at Delft University of Technology in the Netherlands, shares some of Gill's concerns but says without additional information, it's impossible to judge the validity of the new method.

At the moment, scientists don't know the proportion of papers Simonsohn's tool might be applicable to or how sensitive it is. Even if it is very good and wrongly indicts only one in 10,000 papers, it would misfire when

applied widely, says psychologist Eric-Jan Wagenmakers of the University of Amsterdam. And some worry it could lead to witch hunts or score settling between rivals, Nosek says.

Harvard University psychologist Daniel Gilbert says AAAS (the publisher of *Science*) should have top statisticians study Simonsohn's method and recommend how to use it. "If we really do have a new tool to uncover fraud, then we should be grateful," Gilbert says. "But the only difference between a tool and a weapon is in how judiciously they are wielded, and we need to be sure that this tool is used properly, fairly, and wisely."

—MARTIN ENSERINK

GULF OIL SPILL

Researchers Hail New Restoration Program Funds

One of the world's largest oil spills is about to give birth to one of the best-funded ecological restoration efforts ever.

Last week, the U.S. Congress approved, and President Barack Obama is poised to sign, legislation that will set aside for restoration and science projects 80% of the water pollution fines that energy giant BP will pay for its role in the 2010 *Deepwater Horizon* oil spill in the Gulf of Mexico. The fines are expected to total between \$5 billion and \$20 billion. Even the low end will produce "the largest

a curse," predicts ecologist Lance Gunderson of Emory University in Atlanta. "It's going to make real progress possible." But the process is likely to be marked by uncertainty, he warns: "Really big, complex restoration efforts tend to have three phases: a beginning, a muddle, and no end."

Still, having that problem at all is a relief to restoration advocates—including researchers, sportfishing groups, and state officials. As recently as last week, some feared that the bipartisan legislation—known as the

RESTORE Act—would be sunk during an election-year battle over a larger transportation funding bill that includes the oil-spill provisions. But on 30 June, their yearlong campaign to radically change how the government will handle fines for the spill levied under the Clean Water Act ended with celebration. "It's like the dog finally caught the car," says Larry McKinney, director of the Harte Research Institute for Gulf of Mexico Studies at Texas A&M University, Corpus Christi.

Clean Water Act fines are based on the quantity of lost oil and the spiller's culpability. Normally, the money would go to the U.S. Treasury and a special oil-spill fund. But the RESTORE Act will steer 80% of the BP fines back to the five states most affected by the spill: Alabama, Florida, Louisiana, Mississippi, and Texas. It establishes a new Gulf Coast Ecosystem Restoration Council, composed of state and federal officials, which will control 60% of the money. The council must use one-half of that share to carry out a "comprehensive" plan for fix-

ing the gulf's ecological problems, including those that existed before the BP spill. The other half will go to projects nominated by the five states.

Thirty-five percent of RESTORE Act funds will be divided equally among the five states, for use in economic or ecological restoration projects. The remaining 5% will go to two research programs overseen by the U.S. National Oceanic and Atmospheric Administration. One will enable researchers in each state to compete for new "centers of excellence" dedicated to ecological, energy, or economic research. The other will create an endowment for long-term fisheries and ecosystem research.

Officials won't know exactly how much money they'll have until a federal court decides BP's fine. That process could take years if the company can't reach a negotiated settlement with the U.S. government (*Science*, 8 June, p. 1219). Restoration planners, however, aren't waiting to identify potential projects, and McKinney expects that "everyone will try to get their special something on the list." The new council will have to work hard to make sure it doesn't end up funding "a lot of disjointed projects that don't mesh and have an impact on the larger ecosystem level," he says.

In the meantime, Gulf Coast researchers are already receiving money from BP as part of two programs separate from the Clean Water Act litigation. In 2010, BP made a voluntary \$500 million commitment to a 10-year research program that will examine spill impacts. And last year it put a \$1 billion down payment toward what could be another large bill for reversing direct environmental damage from the spill.

—DAVID MALAKOFF



A fine mess. Fines levied for the 2010 *Deepwater Horizon* oil spill will be used to restore Gulf of Mexico ecosystems, such as this oiled Louisiana marsh.

chunk of dedicated money for ecosystem restoration we've ever seen," says Brian Moore, legislative director for the National Audubon Society in Washington, D.C.

The really hard part, researchers say, will be figuring out how best to use the windfall to repair vast wetlands and marine ecosystems battered by more than a century of abuse (*Science*, 14 October 2011, p. 163). Already, scientists and state officials are maneuvering to push their favorite projects to the front of the line. "The money will be a blessing and



TURKEY

Prominent Turkish Academic Who Advocated Secular Reforms Arrested

Until he was arrested, Kemal Gürüz, the former head of Turkey's Council of Higher Education, had led the life of a retired academic for several years, spending most of his time at home reading and stepping out a few times a week to play tennis with friends. Earlier this month, he and his wife, Güniz, a retired chemical engineering professor, were on a cruise along the Adriatic Coast when a friend called to say that Turkish authorities were looking for Gürüz. After reaching shore, he went to a court in Ankara on 25 June. Shortly after, Gürüz was taken into custody and held.

Academics and scientists in Turkey say that Gürüz—a fierce advocate of insulating Turkish education from Islamist influence—has been targeted as part of an ongoing campaign by Turkey's conservative government, which has imprisoned hundreds of progressive military officials, scholars, and journalists. Many are now being tried on charges of having worked for an allegedly terrorist organization called Ergenekon that the government claims is conspiring to destabilize the country. Gürüz himself was detained 3 years ago on a charge of being involved with Ergenekon. His arrest last week pertains to a different inquiry into what's known as the “postmodern coup” of 1997. That year, the military forced a conservative regime to limit religious influence in public life and a few months later pushed the regime out of office.

No charges have yet been filed against Gürüz, colleagues say. “They’ve arrested him as an example to others who are fighting the onslaught of Islamist ideology on Turkish education,” says Ali Mehmet Celâl Şengör, a geologist at Istanbul Technical University in Turkey and a foreign associ-

ate of the U.S. National Academy of Sciences. “They are trying to scare anybody who poses a threat to the Islamist revival in Turkey.” The press counselor at the Turkish embassy in Washington, D.C., had no immediate comment.

Other intellectuals have been targeted as well. Mehmet Haberal, a 67-year-old transplant surgeon of international repute, has been in prison since 2009 on charges of aiding Ergenekon's terrorist activities. “Haberal is, to many people, a real hero,” says Abdallah Daar, a professor of public health at the University of Toronto in Canada and an executive committee member of the International Human Rights Network of Academies and Scholarly Societies, based in Washington, D.C. “Our Human Rights Network has researched his case extensively, and we could find no evidence of him being involved in or advocating for violence.”

Gürüz's supporters and family members believe that his arrest is payback for secularist reforms he instituted as head of the Turkish Council of Higher Education from 1997 to 2003. One change he introduced made it more difficult for students from religious seminaries to get into government jobs that required mainstream education. He also had universities enforce a law that prohibits citizens from making a statement about their religion in government institutions—for instance, by wearing a veil on campus.

Şengör and others suspect that a controversial move by Gürüz while at the council may be at the heart of the matter. Şengör says Gürüz dismissed a university rector named Besir Atalay for violating rules intended to keep religion out of education. Atalay is now Turkey's deputy prime minister. “There is a

Awaiting word. Gürüz and his wife Güniz before his arrest; Güniz says officials have not disclosed why he's being held.

strong element of revenge here,” Şengör says.

The government appears to have had Gürüz in its sights for some years. His earlier detention occurred in January 2009 during a wave of arrests in connection with the Ergenekon investigation. Fifteen policemen entered Gürüz's home in Ankara, seized his computer, and searched the house for several hours, according to Gürüz's family. Gürüz was marched off to a detention center, where his family says he learned that his phones had been tapped for several months. Based on some of the phone conversations, including one with a general, Gürüz was accused of helping Ergenekon.

After Gürüz was released a few days later, he came home and wept, says his son, Murat Gürüz, a materials scientist at Western Digital in Irvine, California. “A lot of people advised him to keep a low profile,” Murat says. But Gürüz did not remain silent. He appeared more than once on TV as a talk show panelist advocating for secularism in education. “My father has a strong personality,” Murat says. “He does not mince his words about what he believes in.”

The government's actions against Gürüz and other academics coincide with what many see as a government effort to populate Turkish academe with sympathizers of the ruling Justice and Development Party. The Turkish Academy of Sciences is feeling the heat. More than 60 members have resigned since last November, when Prime Minister Recep Tayyip Erdoğan announced that the government would appoint new members to the body, which traditionally has elected its own members. Of the approximately 100 new members appointed by the government, several “have absolutely no international publications while many others have no citations,” says Sami Erol Gelenbe, a Turkish-born electrical engineering professor at Imperial College London, who was among the members who resigned.

On 29 June, 4 days after Gürüz's arrest, a court turned down his appeal to be set free. Güniz told *Science* that the authorities have still not indicated when charges might be filed. “We don't have any hopes for the time being,” Güniz told *Science* a few hours after meeting her husband in prison. “We talked on the phone while seeing each other through a glass window. He is very depressed.”

—YUDHIJIT BHATTACHARJEE

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NEWSMAKER INTERVIEW: LAURENCE STEINBERG

Supreme Court Cites Science in Limiting Punishments for Juveniles

Last week, the Supreme Court ruled that mandatory life sentences without the possibility of parole violate the Eighth Amendment ban on cruel and unusual punishment when applied to juvenile offenders. The ruling builds on two other recent decisions, *Roper v. Simmons* (2005), which eliminated the death penalty for offenders younger than 18, and *Graham v. Florida* (2010), which ruled that juveniles convicted of crimes other than homicide cannot be sentenced to life without parole. In all three cases, the court ruled that the sentencing of adolescents should be different from that for adults, in part due to growing research evidence that the adolescent brain is not yet fully developed.

Laurence Steinberg, a child psychologist at Temple University in Philadelphia, Pennsylvania, was the chief scientific consultant on amicus curiae briefs submitted to the court by the American Psychological Association in the *Roper* and *Graham* cases. He played the same role in *Jackson v. Hobbs* and *Miller v. Alabama*, two recent cases involving 14-year-old boys. In one case, Kuntrell Jackson participated in an attempted video store robbery in which another boy shot and killed a clerk. In the other, Evan Miller, along with an older boy, beat Miller's neighbor with a baseball bat and set his mobile home on fire. The man died. Both Jackson and Miller initially received mandatory life sentences, and legal appeals eventually brought their cases to the Supreme Court, which combined them for its ruling last week.

Steinberg discussed the decisions in an interview with *Science*. His comments have been edited for brevity.

—GREG MILLER

Q: Does this new decision represent a big step?

L.S.: I don't think it reflects any huge change in the court's thinking. But it's big in the sense that many more individuals will be affected. With *Roper*, there are actually very few juveniles sentenced to capital punishment. With *Graham*, the number of individuals serving life sentences for nonhomicide crimes committed as juveniles is very, very small. Now, we're really talking about several thousand individuals currently [serving mandatory life sentences] and all of the ones going forward from here.

Q: What have psychology and neuroscience taught us about adolescents that's

relevant to the justice system?

L.S.: Adolescents are significantly different from adults in ways that mitigate criminal responsibility. They're more impulsive and less able to anticipate the consequences of their actions. They're more drawn to the immediate rewards of a decision, and they're more susceptible to peer influence.

Q: That all sounds like stuff that any parent could tell you. Does the neuroscience research actually add anything to the argument?

L.S.: Where I think it's helpful and appropriate is in providing concurrent validation of the behavioral science and helping us understand the neural underpinnings. It's not sim-

ply that there are structural and functional changes in the brain during this time period, it's that those changes map onto what we know about behavioral changes.

Q: Is the research being used in other ways in other courts?

L.S.: The kinds of cases I've been getting called about recently are cases of individuals that are slightly older than 18. A lot of defense attorneys are saying, "Well, if we're going to go by the brain science, the science says the brain is still maturing into their 20s." I don't actually see that going anywhere constitutionally.

Q: The *Miller* case involved a particularly brutal crime. What about accountability?

L.S.: No one has ever said adolescents should be excused from responsibility. All we've argued is that their developmental immaturity should be taken into account for two reasons. One is that it makes them less responsible for their behavior. The second important factor, which hasn't gotten as much coverage, is that they're also in a period of life when they're changing a lot. Most people would look at Miller and say that's just a bad kid. But the research says that even when kids do very violent things at this age, it's still very hard to say with certainty that they're going to continue to be violent.

Q: Do you think the science will ever get to the point where we can predict which individuals are most likely to be violent again?

L.S.: I'd say we're still very far away from that. We did a longitudinal study of 1350 serious juvenile offenders with mostly felony convictions. In our sample, as has been shown in many studies, only about 10% were still repetitive offenders in their early and mid-20s. But if we were to go back and look at the data we collected at baseline, and that was a 4-hour assessment [of many psychological and social factors], we wouldn't be able to predict which ones would be in that 10%. We had so much more information than any court could possibly hope to have, and we couldn't do it. I think the reason is that so many of the factors that lead kids to reoffend are in the environment, not in the kid.

Q: You must be pleased to see your work cited in a Supreme Court decision.

L.S.: It's not just my research—it's a lot of people's. As scientists, we should always be happy when good science plays a role in helping to formulate sensible social and legal policies. It doesn't happen all that often.

Turning Over a New Leaf In China's Forests

A massive redistribution of land-use rights aims to boost afforestation and sustainable forestry

PINGZHANG, CHINA—In this impoverished mountain village of 1680 people, Qian Jinfa's house is like a palace. His clean courtyard home with its sanded wooden doors and freshly painted walls stands in contrast to the grim, single-room cement dwellings of his neighbors here in western China's Yunnan Province. The 42-year-old farmer—whose name consists of three characters meaning money or wealth—took advantage of reforms granting villagers across the nation tenure rights, or limited private use, of forests. Qian leased 5.3 hectares from neighbors—most of whom, like him, are members of the Yi minority group—and combined that with 1.3 hectares of his own land. He then felled about 300 of the tallest pine trees, plowing over \$11,000 from timber sales into his home renovation. “Now that I have a certificate for my land, I can just go to the county seat and sell the trees,” he explains.

Qian's get-rich-quick scheme might sound like a conservationist's nightmare. But for Su Yufang, deputy director of the Kunming Institute of Botany's Center for Mountain Ecosystem Studies, it is precisely the opposite. For decades, much of the forest surrounding Pingzhang, as in other parts of China, was collectively managed. In practice, that meant state-owned timber companies and local officials wielded control—and ecology suffered. To encourage stewardship, the provincial government in 2006 granted villagers the right to

determine the type of forest-use rights they preferred. In Pingzhang, they voted to convert most of the village's forest to individual tenure, allowing households to use plots for 70 years. For Su, the key element in Qian's story is not that he cut down pines, but that he left many trees standing—and that he planted alder, which fixes nitrogen in the soil. Now Qian's main complaint is the recent appearance of squirrels, which eat walnuts on his patch of land. It doesn't concern Su: “That's a sign the forest is getting healthier,” he says.

Last week's Rio+20 sustainable development conference in Brazil pledged to protect the world's forests by promoting secure land tenure. Many conservationists were disappointed that the nonbinding declaration left an opening for conversion of natural forests to industrial use and building infrastructure. But China has already implemented substantial tenure reforms, and no other country's efforts may prove more critical. In sheer numbers, China leads the world in new forest cover: It has added roughly 40 million hectares since the late 1970s, an area roughly the size of Paraguay. Much of that increase has come from plantations of fast-growing Chinese fir and eucalyptus, favored by loggers. Management of older and more diverse forests critical for ecosystem stability, meanwhile, is suffering as the economy booms, demand for timber explodes, and villagers flee to the cities for jobs.

Tenure reforms aim to revitalize China's rural economy while throwing a lifeline to tattered forest ecosystems. The reforms allow villages to determine tenure rights in the roughly 60% of China's forestland that has been collectively owned for decades, affecting about 100 million hectares and some 400 million people. It is the largest action of its kind in recent history, according to a 2010 report from the Rights and Resources Initiative, a nonprofit organization in Washington, D.C. New provisions extend farmers' use rights from 30 to 70 years and permit them to mortgage plots, transfer rights to corporations, and freely make decisions about planting and harvesting.

“To allow the local villagers to think about what they want to do is a very big step forward,” says Heinrich Spiecker, director of the Institute for Forest Growth in Freiburg, Germany. Others caution that the reforms create the potential for forest fragmentation and complicate the task of sustainable management. “The ownership reform has generated a lot of debate,” notes John Innes, dean of the faculty of forestry at the University of British Columbia, Vancouver.

Early signs suggest that the reforms are boosting land productivity and forest cover. In 2011, researchers at Peking University's Environmental Economics Program in China surveyed nearly 300 villages across eight provinces, interviewing 10 to 20 households

CREDIT: M. HYSTENDAH

in each village. The researchers found that timber harvesting in village land managed by individuals had doubled, from an average of 70 cubic meters per village in 2000 to 160 cubic meters in 2010, according to the survey's lead investigator, Peking University forestry expert Xu Jintao. In the meantime, tree planting rose. "That's exactly what you want," says Andy White, coordinator of the Rights and Resources Initiative. "There is growing evidence that people will plant when their rights are secure."

Reclaiming the land

Across the developing world, indigenous groups are pushing for collective forest tenure to gain more control over lands they historically occupied. China's approach to collective rights is a different story. Before Mao Zedong came to power in 1949, forestland in China was largely held by households. After the revolution, historical claims were ignored: "What Mao did was force people to farm collectively," White says.

China's forests suffered. By the late 1970s, large swaths had been stripped of timber. State-owned companies stepped in to manage the remnants, which gave villagers little incentive to look after the land. In Pingzhang, village leader Bi Guang says farmers stood by as the forest became barren, then grazed livestock on the depleted land. "What was everyone's was really no one's," Su says.

In the early 1980s, China began to loosen restrictions, dividing up agricultural communes and granting farmers land rights. Reform-minded officials in the State Forestry Administration, hoping to do the same with forestland, launched an early round of tenure reassessment. Resistance proved fierce. It wasn't just the timber industry, Xu says: "The wood-processing sector, the shipping sector, and the sales sector were all state-owned. If you revised forest tenure, the farmer could have sold timber to anyone. And the industry would have fallen apart." And officials feared villagers might start a clear-cutting frenzy. After a few years of seesawing policies, the reforms were halted.

By the late 1990s, villagers in Fujian, now China's largest timber-producing province, refused to cooperate in conservation efforts. "The system was collapsing," Xu says. The turning point came in Fujian's Hongtian village, where farmers had stripped about one-third of its forest. In 1998, local leaders decided that the only way to protect what was left would be to empower the villagers.

As Hongtian loosened controls, Guangyu Wang, then director of finance and economics in Fujian's forestry department, watched

with interest. He had done a fellowship at the Pacific Northwest U.S. Forest Service; unlike its Chinese counterpart, it did not take orders from the timber industry. Wang and his colleagues "realized the government should separate forest management from the timber business," recalls Wang, now an expert in sustainable forest management at the University of British Columbia, Vancouver. That approach succeeded in Fujian: "More farmers put work into the land," Wang says.

Fujian's reforms later got support from central government officials, who had grown concerned about social instability in the countryside and hoped to promote self-sufficiency in timber. They also realized that healthier domestic forests would be good for China's carbon balance, Spiecker says.



Conservation cash. Qian Jinfa funneled a portion of his timber revenue into tree planting.

Tenure reforms appear to be strengthening forest stewardship. An earlier round of the Peking University survey found that in 2006 and 2007, villages that adopted tenure reform planted an average of 17 more hectares of forest than those that did not. Firewood collection also decreased.

Yet the reforms are expected to create some problems. In dividing forests into smaller parcels, the new policy may complicate fire prevention and other management tasks. Sustainable forestry is "difficult to implement at very small scales," Innes says. Others say that the government must repeal a quota that restricts logging in natural forests, put in place after Yangtze River basin floods in 1998. Although designed to protect

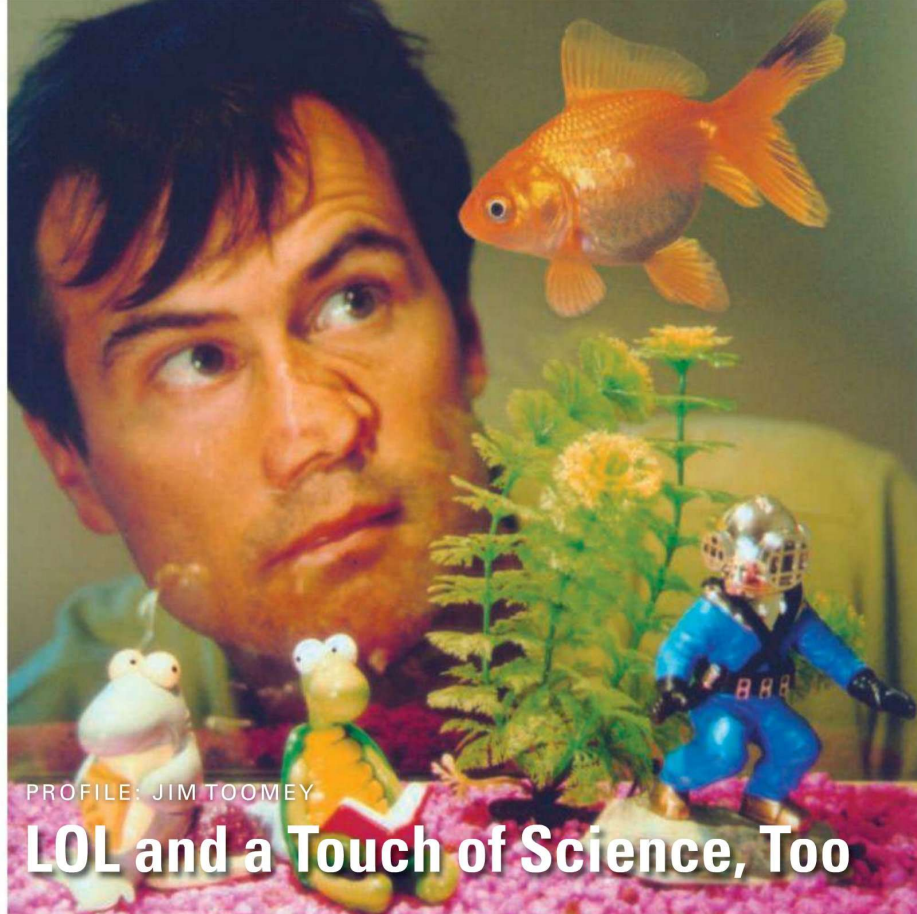
forests, it has curbed enthusiasm for conservation. "If you don't give farmers rights over timber production, then you don't have a lot of incentive for farmers to plant trees," Xu says. Another pressing issue is ensuring that land-tenure rights are protected. Local fiefdoms may brush them aside, leaving room for abuse. For instance, in Guangxi Autonomous Region, Stora Enso, a Finnish paper company, signed a contract with a county government. The county agreed to provide 40,000 hectares of forest by late 2010 that the company would convert to eucalyptus plantations for a paper mill, according to a study funded by the Rights and Resources Initiative and Rural Development Initiatives, a nonprofit in Eugene, Oregon. Local officials then bullied farmers out of their forest-use rights, the study alleges. In one village, armed police showed up with bulldozers, sparking a violent clash.

The spice of life

Leaving villagers to their own devices is fine, experts say, as long as they adopt sustainable forestry. That's a tall order. "For a farmer it is much easier to plant one species," Spiecker says. Eucalyptus and Chinese fir are popular in part because villagers understand the market for their timber, even if they are not wise ecological choices. "That is really an obstacle," Spiecker says. "The individual prefers to have a simple, monoculture forest." To demonstrate the advantages of variation, researchers from the Center for Mountain Ecosystem Studies planted a multipurpose forest just up the road from Pingzhang in 2006. They peppered Chinese pine stands with native species such as candle birch (*Betula luminifera*); *Michelia floribunda*, a type of magnolia; and alder. Today the forest has a dense canopy. "You can tell the soil is better than what's over there," Su says, gesturing to a preserved section of the original forest, which looks like a tree farm.

Multipurpose forests are catching on at experimental stations in China (*Science*, 31 July 2009, p. 556). But convincing locals to carry the torch is a greater challenge. In Pingzhang, leaders discourage planting eucalyptus while encouraging investment in nontimber agroforestry species like walnut. One slogan says, "It's better to have lots of walnuts than lots of sons."

"The key word is education," Spiecker says. Knowledge about sustainable forestry is "available worldwide," he notes. "The question is how to get this information to the people." Small forestry associations could help. But that will come in time, Spiecker says: "All of this has to develop. It cannot be started from scratch." —MARA HVISTENDAHL



Group photo. Cartoonist Jim Toomey posed in 1998 with the characters from his comic strip, *Sherman's Lagoon*.

understated, adds Scott McCloud, a cartoonist and author of several books about comics and their impact. Images can bypass our critical faculties and forge a pretty strong connection in our memories, he says.

Toomey, 51, traces his love of the ocean—and his interest in sharks—to childhood summer vacations spent at Delaware's Rehoboth Beach, a popular tourist destination in the mid-Atlantic region. Fishers casting off a local pier would pull in all sorts of fish, including a few sharks. As he was growing up, the TV specials of marine explorer Jacques Cousteau convinced him that sharks had an undeserved reputation as villainous predators. Flying low over the crystal-clear waters of the Bahamas in a plane that his father piloted, a 12-year-old Toomey spotted a lone shark in a lagoon.

Following the family tradition, Toomey was trained as a mechanical engineer. As an undergraduate student at Duke, he also drew political cartoons for the student newspaper. After college he moved to San Francisco and combined those talents by working at an exhibits company.

After a few years, he decided to use his artistry to tell stories. Thinking back to that Bahamian bay, he created a comic strip with a bumbling great white shark at its center. His friends are all named after streets in San Francisco.

He sent samples of his strips to the top 250 U.S. newspapers, and about 15 signed up. A year later he gave up his day job as an engineer and embarked on a fulltime career as a cartoonist. In addition to penning

Cartoonist Jim Toomey finds ways to sneak marine conservation and science into his popular comic strip

ANNAPOLIS—"Science is hard to make funny," admits cartoonist Jim Toomey. "And I don't want to be known as the guy with science predictably in the story line."

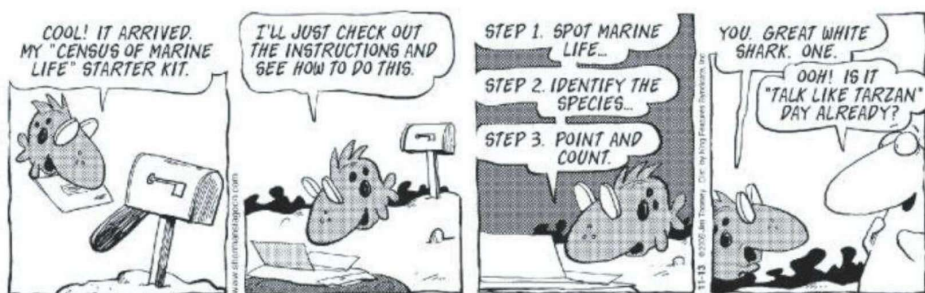
Despite those caveats, the former mechanical engineer has repeatedly used his popular comic strip, *Sherman's Lagoon*, to champion marine conservation. One favorite topic (see panels) is the Census of Marine Life, a decadal effort by scientists to catalog the diversity of life in the oceans. But Toomey, whose strip appears in 150 U.S. newspapers and 25 other papers around the world, has found a way to present the issue without violating the first rule of cartooning: Keep it light.

"I like to dive into scientific themes often enough to keep the audience intrigued," Toomey says. But the more science-oriented the content of the comic, he adds, "the more you whittle down your audience. I'd rather cast a wider net with a thinner scientific message."

For Toomey, humor comes from recognizing oneself in a cartoon character. And his strip offers readers a wide selection of mirrors: In addition to Sherman, an affable great white shark, the strip features a green sea turtle called Fillmore as his brainy sidekick, a scheming hermit crab, and a juvenile-delinquent fish.

Scientists involved in the marine census are basking in the attention that Toomey has given it. "It was a great advertisement to the public at large that really didn't know much about the oceans," recalls marine ecologist Patrick Halpin of Duke University in Durham, North Carolina.

Cartoons offer some inherent advantages for scientists who are trying to reach the public. "Kids put a lot of stock in your strip," says



Stephan Patis, creator of the comic *Pearls Before Swine*, whose characters have opined on quantum mechanics and colliding galaxies as part of Patis's wry and offbeat exploration of the human condition. "You almost teach them how to read, and you are their first introduction to many new ideas."

The visual nature of comics shouldn't be

Sherman's Lagoon, Toomey has lent his talents to outreach programs by the U.S. National Oceanic and Atmospheric Administration, the U.N. Environment Programme, and several conservation organizations. He also serves on the board of directors of the Sylvia Earle Alliance, a group that promotes the formation of marine sanctuaries.

Tradeoffs

Although his characters have often advocated on behalf of scientific causes, Toomey doesn't let science cramp his artistry. Sherman never had his full set of fins, for example, and his sharply pointed nose has become softer over time to the point where it now resembles Snoopy's, the beagle made famous by Charles Schulz in *Peanuts*. Fillmore once had flippers but now has fingers and hands. His shell is more like a sweatshirt than a bony carapace. Hawthorne—besides being enormous for a hermit crab—no longer scuttles like a crab but stands up like a person. “My fish are people in fish costumes,” Toomey says.

Researchers say they are cool with the distortions. “I don't mind that Sherman is a caricature of a real shark,” says marine biologist Demian Chapman of Stony Brook University in New York. “Anything that conveys information about the plight of sharks is something that's really important,” adds Chapman, who studies sharks.

Toomey says it's a constant challenge to make Sherman lovable because a lot of endearing human foibles just don't apply to fish. So forget midriff bulge and receding hairlines. “It's a very narrow slice of the humor pie that you can play with,” he notes.

Toomey is also constrained by the dimensions of the panels of the modern comic strip, which have gotten considerably smaller over time. Whales are too big to interact with Sherman, he points out, while corals are not only too small but also lack a mouth with which to talk. When he wanted to feature an octopus, whose mouths are underneath their bodies, Toomey used a thought cloud instead of a dialogue bubble to convey the creature's views.

Although Pastis says Sherman provides “a natural fit” to explore marine conservation issues, Toomey has learned that readers don't want to be lectured. He once ran a series about the shark-fin trade in which Sherman gets caught, loses his fins, and is tossed back into the ocean to die. After St. Peter tells him that there are too many sharks trying to enter heaven, Sherman returns to Earth and struggles to change his diapers with his teeth. His friends come to the rescue, buying him fins from a Web site and sewing them back on. Although many readers voiced their approval of the series, Toomey

says, some complained that the message was too serious for the comics.

Sherman's adventures

Toomey has taken his cast of characters all over the world. They've skied in the undersea mid-Atlantic Range, seen a 3-meter-wide jellyfish off the coast of Japan, and

numerous Census of Marine Life finds, such as the yeti crab (*Kiwa puravida*), which grows bacteria on its claws to eat; the vampire squid (*Vampyroteuthis infernalis*); and a dumb-octopus (*Grimpoteuthis* sp.).

Census scientists were thrilled. “Scientists can be stodgy and set in their ways and might look down on people doing cartoons,” Halpin says. “But they enjoyed seeing different avenues of communication [for their work].”

Toomey has used his strip to convey a variety of marine science and conservation messages. A plastic bottle floating into the lagoon sends Sherman and his friends on an adventure, tracing the bottle's journey from Boise to the Great Pacific Garbage Patch. When a conservation organization tagged a sea turtle in Cocos Island National Park off Costa Rica and named it Fillmore, the cartoon character took a trip there, and the strip directed readers to the Web site tracking the real Fillmore.

His strip is not the first to tackle marine conservation, admits Toomey, citing the venerable *Mark Trail* as a forerunner. “But *Sherman's Lagoon* is probably the only comic strip that repeatedly revisits the issue.” At the same time, Toomey deliberately puts a spin on the science. “I take the scientific fact and give it a comic twist,” Toomey says.

Take the fact that great white sharks have ampullae of Lorenzini, tiny electromagnetic receptors used to find prey. Toomey turns them into a sixth sense that enables Sherman to win bets on sporting events. Another strip shows Fillmore making a yearly pilgrimage to Ascension Island, where green sea turtles migrate to lay their eggs, for a swinging singles party.

An upcoming series about the Sargasso Sea will include a fish with modified pectoral fins that let it climb up on *Sargassum* seaweed. The comic strip may also propose turning part of the Sargasso Sea into a marine sanctuary, a common theme for Toomey. But some marine topics, such as ocean acidification, are harder to fit into the 20 or 30 words available within a typical strip. “You might see one in a year” on that topic, he predicts.

Anticipating the continued decline in newspaper readership, Toomey has begun writing and producing animated videos in hopes of mastering this potentially more potent medium. Videos and e-books, he says, “will probably be my ultimate future.”

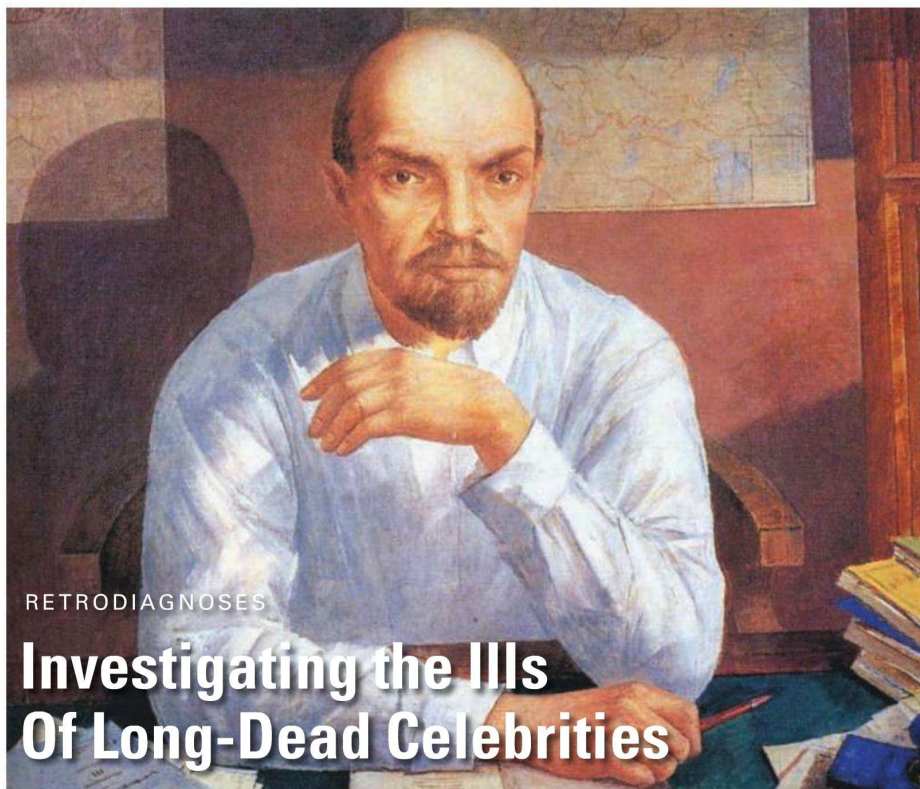
—ELIZABETH PENNISI



descended to the ocean's greatest depth in the Marianas Trench. “What I am trying to do is bring some of the ocean to the public that they might not be aware of,” Toomey says. “I'm fascinated with the oddballs.”

The Census of Marine Life is a natural stage for oddballs. Halpin had met Toomey while the cartoonist was getting a master's degree in environmental management at Duke. “It was really interesting to have someone not only doing a cartoon on marine life but also dedicated enough to the field to get his graduate education in it,” Halpin says. After a colleague suggested recruiting artists to help get the word out about the census, Halpin e-mailed a request to Toomey. The cartoonist saw great potential in the idea.

“I turned it into what the human census was because that's what people could instantly identify with,” Toomey says. As a result, Ernest the fish gets his census-taking kit in the mail and goes door-to-door looking for new critters (see panel). He comes across



RETRODIAGNOSES

Investigating the Ills Of Long-Dead Celebrities

Vladimir Lenin's death was examined recently by a medical group that tries to decide how historical figures died; some historians disapprove of the practice

BALTIMORE, MARYLAND—Vladimir Lenin had a rough last few months. Although just 53 years old, by 1924 he'd become aphasic, unable to speak much more than monosyllables such as *vot-vot* ("here-here"). His right side had become virtually paralyzed, and he suffered convulsions. Moreover, he was paranoid about someone poisoning his food. Lenin had good reason to be paranoid: As his health dwindled, the shadow of Joseph Stalin was looming ever larger over the Soviet Union.

Online
sciencemag.org

S Podcast interview with Sam Kean (http://scim.ag/pod_6090).

Lenin died on 21 January 1924 after an apparent seizure. Doctors have rehashed the event for decades, proposing any number of causes: syphilis, strokes, heart disease, lead poisoning from the bullets that lodged in Lenin's body after an assassination attempt. Doctors even preserved Lenin's brain in a jar to study it, but no one has ever reached a firm diagnosis.

Far from deterring interest, the murky circumstances of Lenin's death have made it popular among medical history sleuths. The most recent group to take it on was the 19th annual Historical Clinicopathological Conference (HCPC) here in May. The HCPC examines

cases of historical celebrities who suffered from mysterious ailments, such as Ludwig van Beethoven, Christopher Columbus, and King Herod. Diagnosing ancient emperors and explorers may seem an eccentric pursuit for doctors—the “patients” have long since died—but the HCPC is hardly alone. The quest to retrodiagnose dead celebrities has proved irresistible to doctors for over a century—although some historians wish that doctors would stop trying to revise history and let the dead rest in peace. Some also dismiss the work as unilluminating and note that DNA testing of the dead can cause distress among living descendants.

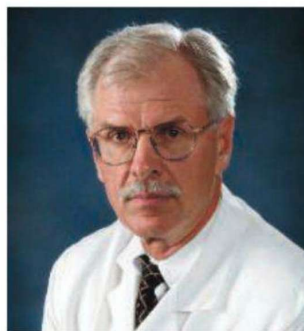
Philip Mackowiak, an epidemiologist and professor at the University of Maryland School of Medicine in Baltimore, dreamed up the HCPC after reading about the wretched final days of Baltimore's own Edgar Allan Poe. Mackowiak had always loved the interactive nature of traditional clinicopathological conferences, in which participants get a case study a few weeks in advance, allowing them to sort through the

Strange seizures. Vladimir Lenin had convulsions before death, suggesting poison to some.

patient's symptoms, arrive at a diagnosis on their own, and then compare their reasoning with an expert's. Mackowiak decided to hold an informal conference on Poe without telling anyone they were studying a man who died in 1849. The forum proved such a hit that Mackowiak made it an annual event.

The HCPC's mystery-solving record, like that of historical retrodiagnoses in general, is mixed. In some cases, as with the ancient pharaoh Akhenaten (HCPC 2008), so few records have survived that achieving a firm diagnosis has proved impossible. More recent cases, such as civil rights leader Booker T. Washington (HCPC 2006), were noted successes. Some historians had long argued that Washington died in 1915 of syphilis, but in 2006 Mackowiak helped obtain hospital records for Washington that listed a negative Wasserman result, the standard test for syphilis, putting speculation to rest.

Then there was Poe. After he died and his literary reputation soared, his doctor began a lucrative lecturing tour during which he denied what most people suspected—that Poe drank himself to death. Building on that doctor's descriptions of symptoms, a speaker at the 1995 HCPC concluded that Poe died not of alcoholism but of rabies. This juicy speculation—how fitting for Poe to die of something so lurid—got a lot of media attention (*Science*, 27 September 1996, p. 1805) and even appeared in a *Final Jeopardy* question in 1997.



Diagnosis hunter. Philip Mackowiak started the Baltimore study group.

When Mackowiak revisited Poe's history for a book he was writing, however, he realized that Poe's doctor had exaggerated some symptoms and downplayed others, making the rabies diagnosis untenable. “There's a tendency” in historical medicine, Mackowiak says, “to want to give extraordinary people extraordinary diagnoses.” But in the end, he says, Poe probably

did die of alcoholism.

Medicine versus history

This year's Lenin talk took place in an old-fashioned amphitheater with wooden seats. Busts of Plato, Zeus, and Homer oversaw the proceedings from a balcony.

The first speaker, Harry Vinters, a pathol-

ogist and stroke expert at the University of California, Los Angeles, Medical School, had never delved into historical medicine before. He first read Lenin's autopsy report as an appendix in a biography, and although the autopsy used strange terminology, Vinters found it thorough and well-done. (Autopsies started becoming common in the 1600s, but only became scientifically reliable around 1900, when doctors had better medical knowledge and started including not only what they found but also what they did not find.)

Based on Lenin's symptoms and autopsy, Vinters became convinced that Lenin suffered multiple strokes. Vinters acknowledged in his talk that Lenin lacked some risk factors for strokes: He didn't drink and, unusually for a Russian radical, abhorred smoking. Moreover, he probably didn't have high blood pressure, because his kidneys looked normal and his heart wasn't enlarged.

On the other hand, Lenin's risk was increased by his stressful lifestyle and a family history of vascular trouble. His father died, probably from a stroke, at age 54. And Lenin's cerebral arteries were so calcified after his death that, when struck with a tweezers, they rang like stone. Overall, given the aphasia, paralysis, and other symptoms, Vinters diagnosed ischemic infarction in Lenin, a death of brain tissue due to narrowing of the cerebral arteries.

But did the infarction actually kill Lenin? The conference's other speaker, Lev Lurie, a historian in St. Petersburg, Russia, thinks not. By 1922, Lenin had already called Joseph Stalin the Communist Party's biggest menace, and Lenin planned to squeeze Stalin out. But as Lenin was slowed by ill health, Stalin was plotting to seize power. And poisoning Lenin, Lurie says, would have completed Stalin's coup. In support of this, Vinters noted that stroke victims normally don't suffer convulsions, as Lenin did. Virtually any poison can cause them.

Beyond pinning down the cause of death, part of the fun of any retrospective diagnosis is the counterfactual speculation it provokes. Had Lenin not died, "Stalin easily could have been driven out of the party," Mackowiak notes, and the Soviet Union would have evolved quite differently. (Vinters can attest to this personally: His grandparents fled from Latvia to North America only after Stalin seized Latvia in the mid-1940s.)

Science historians tend to shun overt

speculation, though, says Axel Karenberg, a medical historian at the University of Cologne in Germany. Karenberg calls himself a former "diagnosis hunter." While a medical student, Karenberg wrote a thesis diagnosing a favorite pianist, Frédéric Chopin. He concluded that Chopin died of tuberculosis and not cystic fibrosis, as one popular theory has it. After switching careers to become a historian, though, Karenberg realized that doctors often fail to meet the best standards of evidence when they venture into historical territory. He began discouraging the practice.

"What [doctors] usually do is read a biography and extract some passages dealing with medicine and come up with a [theoretical] diagnosis," he says. They do something similar with living patients, he acknowledges, but with living patients, physicians can also follow up hunches with tests to prove diagnosis A and rule out diagnosis B—an important aspect of scientific medicine but impossible with dead celebrities. Moreover, doctors often draw on dubious sources to make historical diagnoses, such as deathbed accounts from laypeople or sources compiled hundreds of years after death. Karenberg

also faults journal editors for accepting and publishing weak papers: "They would never publish a paper of this level of speculation on cancers or strokes," he says.

Perhaps most important, Karenberg argues, retrodiagnoses often miss the point. Doctors might well pin a correct diagnosis on a famous dead person, he says, but a historian would look instead for "how the disease altered his life or mental state. The label is unimportant." If retrodiagnoses teach us anything, Karenberg says, it's about the practice of science itself. Usually when scientists discover a new syndrome, people will start retrodiagnosing historical celebrities with it within about 10 years, he says. "What you're getting is a perfect mirror of the history of medicine and of what diagnoses came into use."

One modern tool that increasingly gets

incorporated into retrodiagnoses is genetics: For instance, scientists recently proposed exhuming Chopin's heart (interred in a pillar in a Warsaw church) and testing for cystic fibrosis mutations. But although genetics can sometimes provide solid historical

evidence—work published in 2010 on King Tut and other pharaohs shed needed light on his family dynasty—genetic testing of the dead can also introduce ethical quandaries because test results may affect the living as well, says Jordan Paradise, a law professor at Seton Hall University in South Orange, New Jersey.

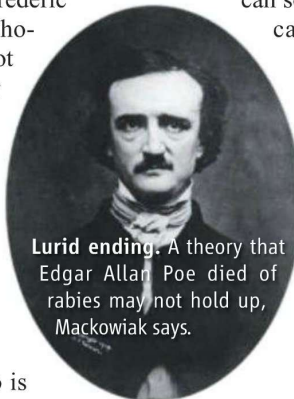
Paradise began studying genetic testing when she was a law student in Chicago advising the Chicago History Museum. It houses numerous artifacts stained with the blood of Abraham Lincoln (a 2007 HCPC subject). The museum had received requests to permit DNA testing of Lincoln's blood for certain genetic disorders. In the end, it turned them all down.

That's partly because such tests destroy small bits of the artifacts but also because of privacy concerns. The dead cannot say no, and genetic tests can expose personal details that no one at the time could have known or understood. And if the person has descendants, DNA tests could reveal health problems or questions about paternity lurking in the genes, things living relatives may not want revealed.

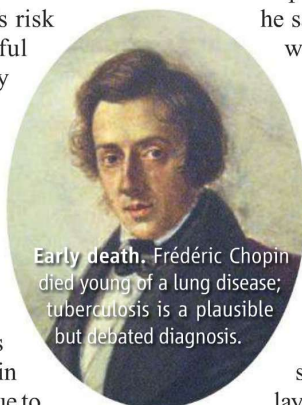
Paradise says that most of the HCPC's work—such as pinning down a diagnosis by parsing old autopsy reports—seems less troubling. "You're just looking back at someone else's account," she notes, not "inserting yourself into that time."

No matter how inconclusive nailing down diagnoses is, Mackowiak says the study of medical history is worthwhile. It provides a fun escape for doctors, he says, and gives them perspective on science history. Above all, he notes, piecing together a retrodiagnosis "generates humility, which is essential for physicians." As he explains, "Every generation thinks it's finally found answers to life's great mysteries, but [in retrodiagnosing people] you come to realize that future generations will be just as dismissive of our efforts."

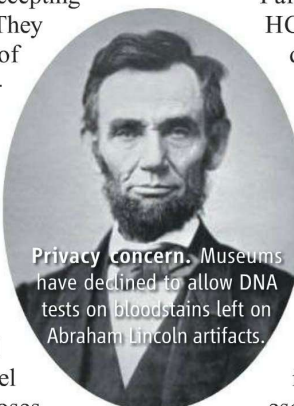
—SAM KEAN



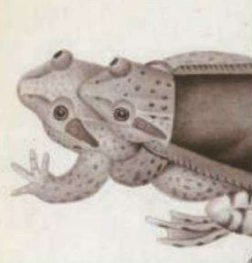
Lurid ending. A theory that Edgar Allan Poe died of rabies may not hold up, Mackowiak says.



Early death. Frédéric Chopin died young of a lung disease; tuberculosis is a plausible but debated diagnosis.



Privacy concern. Museums have declined to allow DNA tests on bloodstains left on Abraham Lincoln artifacts.



LETTERS

edited by Jennifer Sills

NextGenVOICES

Results: Experiences That Changed Us

In April, we asked young scientists to describe a specific experience and how it changed their science, training, or career goals.

We heard from more than 100 readers. A sample of the best responses can be found below. To allow for as many voices as possible, in some cases we have printed excerpts of longer submissions. To read the complete versions, as well as many more, go to <http://scim.ag/NextGen3Results>.

Submit Now: Big Ideas

Add your voice to *Science*! Our new NextGen VOICES survey is now open:

What one big idea in your field do you wish that every non-scientist understood? Why?

To submit, go to http://scim.ag/NextGen_4.

Deadline for submissions is 17 August. A selection of the best responses will be published in the 5 October issue of *Science*. Submissions should be 250 words or less. Anonymous submissions will not be considered. Please submit only once.

most to the development of neurons into various types? Why does the body set exact circuits in balancing diversified feedback loops? How do multiple mediators act in concert to produce an orchestrated “symphony” that enables the body’s responses to various challenges? Biology is amazingly beautiful!

WEN-QIANG CHEN

Department of Physiology, Li Ka Shing Faculty of Medicine, University of Hong Kong, Hong Kong, China. E-mail: chenwq@hku.hk

NextGen Speaks

AIR FRANCE FLIGHT 3210 PARIS-NOUMEA. I am taking off for my first field season and, like any neophyte Ph.D. student, I have brought with me an ambitious pile of articles, in order to optimize my 24 hours on the plane. Although my thesis is focused on mathematical models of trophic relationships on islands, the scope of my literature selection is broader. “Stable isotopes reveal evidence of predation in seabirds on the Shiant Islands, Scotland” (Stapp 2002)—the title frightens me a bit; chemistry is an uncommon component in the methodological mixture of fieldwork that will start in 2 days! In the end, intrigued, I decide to add stable isotope sampling at the last minute. Little could I have anticipated how this article would enrich my future career. Ten years later, stable iso-

topes are part of my daily routine and compose the core of my research. What would I be doing, who would I be, if I had set that pile of articles aside and nestled down into my seat, like the other 250 passengers, to watch the in-flight movie?...

STEPHANE CAUT

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IN MY SCIENTIFIC CAREER, MOZART IS THE ONE who inspires me constantly. When I was a teenager, I was absorbed by Mozart, because his music is so pure and concise. When I began to study biology in my university, I felt the same purity and conciseness: Our body functions as elegantly as Mozart’s music does! ... I am always wondering: What is the power determining cell fate? Which factor contributes

... IN MY THIRD YEAR OF UNDERGRADUATE study, I went to Japan on an exchange program, and in order to get enough credits I joined the immunology laboratory for a research project. Everything in that lab—the techniques, the equipment, the experiments—was so new to me. The project I was given involved expression of some recombinant protein in *E. coli*. This particular protein is quite difficult to express, and after many weeks of trying I just could not approach my supervisor and tell him the experiment had failed AGAIN!!! I felt so overwhelmed that I went to the darkroom and started crying. A colleague of mine found me, and after I had explained the situation to her, she explained to me that every single scientist has had to go through many such moments. At that point, it hit me: For all the things I read in textbooks, someone at some point went through exactly what I was going through. I had never reconciled those two facts so solidly together. Until then, my sole purpose had to be to get good grades ... then and there in that darkroom, I decided to be a biomedical researcher when I returned to my country.

ANNE WANJIRU NDUNGU

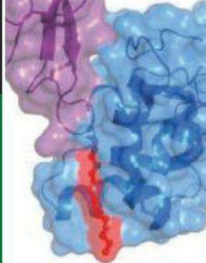
Kemri-Wellcome Trust Research Programme, Immunology Laboratory, Kilifi, 80108, Kenya. E-mail: awanjiru@kemri-wellcome.org





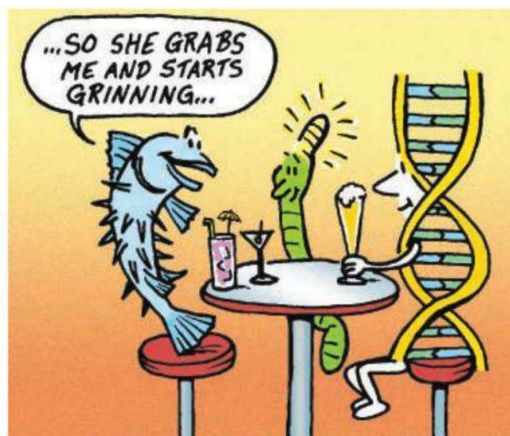
Pyruvate transporter identified

41



Ligand-receptor interactions

44



AS AN UNDERGRADUATE I APPLIED TO AN internship at Mote Marine Laboratory, hoping to work on a marine mammal project, but I was assigned to a dolphin prey species survey instead. Initially, I was bummed. I wouldn't get to work with dolphins, but would instead be fishing all summer. On my first day on the boat, I was regaled with all the very important safety procedures and got an extensive lesson on purse seining, where you use a large mesh net to sample the water column. We motored out to our first site and set the net. I was poised; ready to measure, ID, and count the fish as they came up. What I didn't realize

was how thrilling it would be. I distinctly remember grabbing my first pinfish. I was pricked and prodded by its spines as I tried to get the slippery, flapping fish to lay flat for a good measurement.

It was at that moment when I realized, "Aha! This is what I want to do!" I marveled at the diversity of sizes, shapes, colors, and even sounds of these animals. From that point on, I knew I wanted to study fish; how they work and interact with their environment. I made my decision that summer to pursue a master's degree in fish physiology, and now as I prepare to graduate, I am so thankful I had the opportunity to spend a summer fishing....

GENINE K. LIPKEY

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I STILL REMEMBER HOW BEAUTIFUL IT LOOKED on the crinkled pages of my ninth grade biology textbook. Behind the bursting red nucleus was forked DNA unraveled from its usual, beautiful helical shape. As an mRNA was produced, it threaded through the open pores of the nucleus and into a ribosome. Even after school ended, these artistic illustrations remained animated in my mind. I wondered what color the nucleus really was and

how these operations could occur so quickly, intricately, and flawlessly in our millions of cells to produce an organism so complex and diverse.... At this point, I knew that I wanted to pursue a biologically relevant field as a career....

LUVENA ONG

Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA. E-mail: luvena@mit.edu

ONE AFTERNOON WHEN I WAS 14 YEARS OLD, I saw a very peculiar fluorescent worm.... This event changed my entire life. Since that day, everything about nature was interesting to me, and I got involved in science. Now that I am in the biological sciences, I know that the bioluminescent worm I have never seen again is one of the forces that makes me fight to become a researcher.

RIGOBERTO MEDINA ANDRÉS

Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad Autónoma del Estado de Morelos, Cuernavaca, Morelos, 62209, México. E-mail: medina-ar@live.com

DURING MY FORMATIVE YEARS, I TRAINED AS an associate at a medical research institute in-house at a leading hospital. Generally, when patient tissue samples were accessed from the hospital facilities, it was always anonymous and alphanumerically coded. On one occasion, the family of a deceased patient with a unique medical case history refused to grant access to the mortal tissue remains for research use. A personal assurance from the director finally helped convince the nearest kin.

As a follow-up, they were requested to attend one of our research meetings where we discuss results from those very samples. The wife and the teenage son sat in one corner of the seminar room, gazing nervously at the scientific populace. They looked through all the histopathological and immunofluorescence images, perhaps understanding nothing, but trying to find which slide they perhaps had a bloodline tied to. As I escorted them to the lift lobby after the meeting, the lady held my hand in reassurance and gratitude, with glistening eyes pouring over with faith.... Since then, I have seen thousands of cell and tissue slides. And every single time I think of her gaze and her unspoken words when I finally place the cover slip over the

MY CAREER WAS DEFINED BY A SHORT ARTICLE written for high school biology teachers in the late 1970s. I was a sophomore in high school in San Juan, Puerto Rico, at that time. Biology was that one class that felt true to me in that awkward and social-pressured time called high school, and I quickly became very interested in the class and in nature itself. During off times, like at lunch or immediately after the last bell, I would wander into the biology lab and spend time with the biology teacher and other like-minded students. There was always something cool to watch, like the fish tank with the sea horse that one of my classmates brought in or the assembly of a cat skeleton in progress. One afternoon, I picked up a short periodical among my teacher's pile of papers and read an article describing the cloning of the insulin gene by scientists at Genentech in California. The article described how researchers cloned and produced large amounts of recombinant insulin in *E. coli* without this being a detriment to the cells. The bacteria had become a mini protein factory that could in theory eliminate the need for pig stomachs as a source of insulin for diabetics. After reading the article, I thought to myself "This is what I want to do." There was no question. I have yet to waver from this path....

JANET F. STAAB

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TECHNICAL COMMENT ABSTRACTS

Comment on “Impacts of the Cretaceous Terrestrial Revolution and KPg Extinction on Mammal Diversification”**Olaf R. P. Bininda-Emonds and Andy Purvis**

Meredith *et al.* (Reports, 28 October 2011, p. 521) question three findings of our delayed-rise hypothesis for present-day mammals made with reference to the Cretaceous-Paleogene (KPg) boundary, based on their new time tree of the group. We show that their own data do not support their objections and that the macroevolutionary patterns from the respective phylogenies are not statistically different.

Full text at www.sciencemag.org/cgi/content/full/337/6090/34-a

Response to Comment on “Impacts of the Cretaceous Terrestrial Revolution and KPg Extinction on Mammal Diversification”**William J. Murphy, Jan E. Janecka, Tanja Stadler, Eduardo Eizirik, Oliver A. Ryder, John Gatesy, Robert W. Meredith, Mark S. Springer**

Bininda-Emonds and Purvis reanalyzed our mammalian phylogenetic supermatrix and claim that our results are not significantly different from their delayed-rise hypothesis. We show that our divergence times are ~11 million years later for placental inter- and intraordinal divergences—consistent with a post-Cretaceous-Paleogene (KPg) radiation of most modern mammalian orders—and find no support for the early Eocene delayed-rise hypothesis.

Full text at www.sciencemag.org/cgi/content/full/337/6090/34-b

sections. I wouldn't say the feeling makes my images look amazingly good, but it gives me that extra willpower and hope to regard my efforts as precious.

KINGSHUK PODDAR

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... IF I HAVE TO NAME ONE EXPERIENCE THAT changed my career goals, it was a visit to a rural high school outside of Winston-Salem, NC. I was there for NHGRI's National DNA Day. The teacher told us that he had to teach evolution at the end of the semester because

parents were bound to pull students out of class. If he taught it earlier, he would lose the student for the entire semester. I was shocked and saddened to hear this. As long as people who live so close to where I practice science feel this way, I won't be content to only pursue research—science outreach will always be some part of my career.

VIRGINIA K. HENCH

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I WAS LEADING A BIOLOGY 101 EVENING LAB, and one of my older students was having a little trouble with the microscope. I helped her find the elodea leaf and then put the specimen in focus. When she looked again, she yelled out, “I see it, I see it, I see it!” and did a little



dance. She was so excited and thanked me over and over again for my help. That moment will always stick with me; at that time I wasn't completely sure I would make it as a great teacher.

I think I am a good teacher, but I know I can be better. That one moment helped focus me to work harder to have more moments like that! I want people to see the science all around them and to help them understand it better!

CHRIS WAYNE HOLMAN

Division of Math and Sciences, Department of Biology, Piedmont Virginia Community College, Charlottesville, VA 22902, USA. E-mail: cholman@pvcc.edu

I WAS BORN AND RAISED IN A VILLAGE IN NEPAL. During my high school time, only talented students were encouraged to major in science. Most of the community members believed that studying science leads to a better job and good life in the future. This is true. That community perception actually brought me to science.... Now I am happy with a career in chemistry....

BASANT GIRI

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JUST OUT OF COLLEGE WITH A JAPANESE LITERATURE degree, I toured a state-of-the-art recycling plant in Yokosuka, Japan. When I heard the figures on the staggering amount of waste the facility processes (about 220



tons per day, or around 50,000 tons per year for a city of 400,000 people, or around 125 kg per year per person), I was amazed; it seemed like a lot of trash

for a small city. This was what started my investigation of the fate of household waste. Then I got into looking at where the things we use come from, which eventually made me realize it was time for a career change. It turns out you need to know things like physics, chemistry, and biology to understand energy and material cycling, so I went back to school. I ended up studying agriculture and environmental chemistry, and that's what I'm up to today.

MIKE MASSEY

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WHEN I WAS 18 YEARS OLD, I WORKED IN A hospice for 12 months as part of my civilian service requirement of the German government. I accompanied old and young people in what was the last chapter of their lives....



Throughout my time in this hospice, I learned many life lessons from older individuals about the importance of changing priorities when you get older, “letting things go,” and the value of time

over money. Some of the merits of these lessons I only now begin to realize. These and other experiences inspired me to study the psychology and neuroscience of aging, because I realized that the aging mind and body has a fascinating recovery potential and experience that is often so underutilized. Sometimes I am still stunned by the variety and diversity of the aging population, and although I see less of them in my daily routine, I am grateful for the opportunity to study the aging mind.

GÉRARD NISAL BISCHOF

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Letters to the Editor

Letters (~300 words) discuss material published in *Science* in the past 3 months or matters of general interest. Letters are not acknowledged upon receipt. Whether published in full or in part, Letters are subject to editing for clarity and space. Letters submitted, published, or posted elsewhere, in print or online, will be disqualified. To submit a Letter, go to www.submit2science.org.

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ECONOMICS

It's the Politics

Geoffrey Garrett

Why some countries are richer than others has long preoccupied some of the world's great minds. Their answers reflect two schools of thought. Some, like Jared Diamond, think geography is destiny. Others believe human agency is the root cause of success and failure. *Why Nations Fail* falls squarely in the latter category, but in a novel way. Adam Smith argued that countries that follow free market principles do better. Max Weber thought a cultural work ethic was needed to make the market work. The book's fundamental proposition is that while markets and culture matter, both, in the jargon of social science, are "endogenous" to the political system.

Put simply, Daron Acemoglu and James Robinson argue that free markets and a culture of enterprise and innovation only thrive in political systems that are open and "inclusive" (of anyone who wants to participate). In contrast, countries run by a narrow elite will fail, because the elite will entrench "extractive" practices that benefit them at the expense of their societies as a whole. In turn, development trajectories tend to be path dependent. Once a country is on a good or a bad track, it is hard to change because of the powerful interests invested in the status quo.

Acemoglu (an economist at the Massachusetts Institute of Technology) and Robinson (a political scientist at Harvard) see England's 17th-century Glorious Revolution not only as the dawn of democracy—it also made possible the Industrial Revolution. America's revolutionaries not only evicted the monarchy. They also created an economic system that made Apple and Google possible.

In contrast, the authors argue, Mexico's extractive political system has committed it to enduring poverty—at least relative to its northern neighbor. Half a millennium ago, Mexico's Spanish colonizers put in place a political system in which a small, unaccountable, and corrupt elite pillaged the country's rich natural resources for its own benefit at the expense of the country's long-term development. Acemoglu and Robinson believe it is the political border between the United States and Mexico, not any other differences, that

explains the two countries' radically divergent development trajectories. The several inches difference in average heights between North and South Koreans offers an even more striking example of their politics-matters thesis.

More generally, Acemoglu and Robinson contend that political institutions laid down in the 16th and 17th centuries have had enduring economic effects. The British and French democratic revolutions were the "killer app" that transformed a struggling and warring northern Europe into an economic titan and made possible the successful Anglo new worlds of America, Australia, and Canada. Whereas when Spain's imperialists found gold and other valuable commodities, they entrenched political systems designed only

to extract resources, not to develop new economic opportunities.

The authors can't scientifically test their bold and sweeping thesis because one can't experiment on world history. Instead, they embark on a journey through history that is breathtakingly sweeping and erudite as well as reasoned and reasonable.

How convincing is their narrative that, to turn the famous Bill Clinton aphorism on its head, "It's the politics, stupid"? Adherents of other big-picture approaches are unlikely to be persuaded, because history is overdetermined. Mexico, for example, suffers from the problems of many tropical countries, such as poor water, crops that perish, and rampant disease. Its brand of Catholicism is light years from Weber's protestant work ethic. And its economic

policies would have Adam Smith scowling in his grave. The continental United States is more temperate, more Protestant, and better governed economically. In this rendering, political variables are superfluous.

But there is a bigger challenge to the Acemoglu-Robinson thesis—the fact that today's economic world doesn't seem to fit their model at all neatly. For example, Greece's economic problems seem to stem from too much political inclusion, not too little. People who worry about India's economic future believe the subcontinent's major problem is too much democracy.

China's three decades and counting economic miracle is an even bigger prima facie challenge to their argument, and Acemoglu and Robinson know it. That is why they spend the last part of their book reinterpreting the Middle Kingdom. According to them, China has grown so spectacularly not because its post-Mao leaders have been benign authoritarians who have used the levers of the state to drive development. Instead, they argue that China has been able to grow at 10% a year for 30 years because of a propitious combination of demography and development. China's rapidly growing working-age population—turbocharged by urbanization unlike anything the world has seen before and coupled with the poverty in which most of the country was mired before Deng's reforms in the late 1970s—has given the country a massive pool of low-cost labor that it has parlayed into becoming the world's most competitive assembler and manufacturer.

Because of the one-child policy, China's labor force has already peaked and will soon begin to decline. At the same time, China is

Why Nations Fail

The Origins of Power, Prosperity, and Poverty

by Daron Acemoglu and James A. Robinson

Crown, New York, 2012. 541 pp. \$30. ISBN 9780307719218. Profile, London. 464 pp. £25. ISBN 9781846684296.



Test case: 21st-century China (here, Shanghai).

increasingly less a low-cost producer than a country of middle-class consumers. How will China continue to grow when it is an aging middle-class country? Here Acemoglu and Robinson argue that unless it becomes a more inclusive political system, China will soon become mired in the “middle income trap.” They don’t say inclusive political change is inevitable—quite the opposite, given their faith in the path dependency of vested interests. But they do make the big call that absent real political reform, China’s economic miracle will end.

Acemoglu and Robinson may well be proved right about China, and perhaps that will convince skeptics of the power of their politics-matters-most thesis. Regardless, their fascinating and thought-provoking book should be read, discussed, and debated far beyond the ranks of social scientists.

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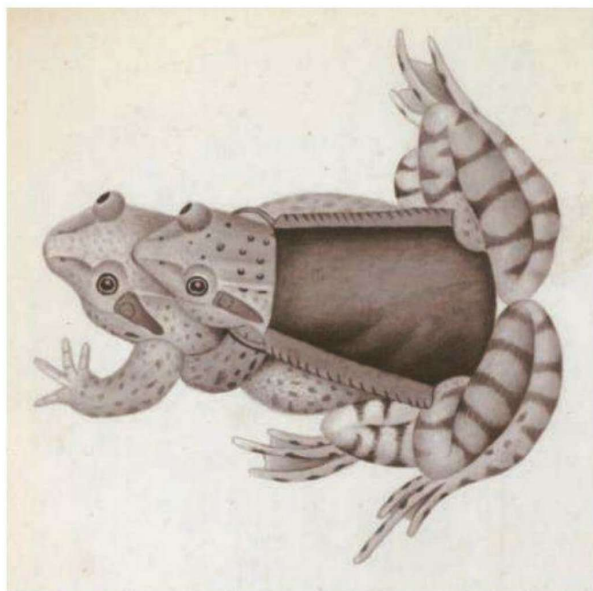
HISTORY OF SCIENCE

Looking to Learn

Carla Nappi

“Frog pants.” When I tell people about *Histories of Scientific Observation*, which is usually in the context of telling them that they need to read it, this is what I lead with. If a physical copy is at hand, I turn open the pages to the image of a male frog wearing waxed taffeta pants (with shoulder straps) as he embraces his mating partner while the two of them look serenely into the distance. This, alone, is worth the price of the book. As it happens, however, there is much else to recommend a careful reading of the volume.

The editors’ introductory overview explains the rationale for and organization of the book. The first three chapters introduce the basic contours of the history of scientific observation from 500 to 1800, with the authors focusing on the origins (Katharine Park), emergence (Gianna Pomata), and consolidation (Lorraine Daston) of observation practices in medieval and early modern sciences. The remaining 14 chapters are arranged in four sections, each cohering around a cen-



Mating frog with taffeta trousers. Hélène Dumoustier de Marsilly’s drawing, documenting experiments carried out in the 1730s by the French naturalist René-Antoine Ferchault de Réaumur. These tested whether the male’s gripping thumbs were the means of fertilization.

tral component of the practice of observation in history: evidence, new techniques, new objects, and communities. Daston and Elizabeth Lunbeck carefully preface each set of essays with a short overview that explains the grouping and summarizes each of the essays within the section, making it easy for readers to understand how the essays fit into the larger narrative of the volume.

The majority of edited volumes published in the social sciences and humanities, certainly most that I have come across in several years of reading them, are chimeras. A lion’s head here, a serpent’s tail there: a Frankensteinian patchwork bandaged together by the memory of a conference and a dutifully composed editorial introduction. The pressure on conference organizers to promise funders a published volume as a material outcome of a scholarly gathering has left the academic world overrun by collections of essays that often would be better suited to publication in journals than bound in hardback. Blissfully, this is not one of those.

Despite the breadth of its contents, the volume is remarkably cohesive, with many of the chapters explicitly cross-referencing others and making one feel as if the authors are inviting us into an ongoing conversation. Indeed, *Histories of Scientific Observation* is the result of a sustained engagement among the editors and contributors that extended

across several meetings over several years. The essays represent an extraordinary range of materials, topics, and case studies within the history of the sciences. They explore contexts from the Middle Ages through the late 20th century and chart the ways that the practices and language of observation evolved along with the emergence and transformation of scientific fields of inquiry. These components make sense as parts of a structure that was carefully planned from its inception: they were born together, grew together, and inform one another. This is a model of what an edited collection can be.

There is no way that a single volume, even one as multifaceted as this, could give a

comprehensive history of observation in the sciences, and the editors wisely make no such promises. In choosing such varied case studies, however, they have achieved something that is an important contribution to the history of science: the essays stand as exemplars of many, many different approaches to understanding the same problem. These approaches include a careful study of archival documents on 18th-century French natural history (Mary Terrall and the frog pants); a spirited and often humorous account of scientific attempts to understand blushes on 19th-century faces and erections in 19th-century trousers (Otniel Dror); and a fascinating exploration of empathy in 20th-century psychoanalysis (Lunbeck). Most of the authors wisely choose a very limited case (perhaps the work of one or two major figures) to probe much larger epistemological issues, but aside from this commonality the sources and historiographical approaches of the contributions are exceptionally diverse.

Visual studies constitutes an enormous field. There are countless works available on varied aspects of the history, theory, creation, and interpretation of images and an entire subfield devoted to the study of images and visibility in the sciences. All of this makes the contribution of *Histories of Scientific Observation* even more remarkable. There is nothing else like the volume currently available, and it should be required reading for both historians of science and scientists interested in the history of their craft.

Histories of Scientific Observation

Lorraine Daston and Elizabeth Lunbeck, Eds.

University of Chicago Press, Chicago, 2011. 472 pp. \$75, £48.50. ISBN 9780226136776. Paper, \$27.50, £18. ISBN 9780226136783.

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RESEARCH ETHICS

Paying Patients for Their Tissue: The Legacy of Henrietta Lacks

Robert D. Truog,^{1*} Aaron S. Kesselheim,² Steven Joffe³

In *The Immortal Life of Henrietta Lacks*, Rebecca Skloot tells the moving story of the woman who was the source of the first immortal cell line (HeLa) (1). The cells were obtained at Johns Hopkins University in 1951 from biopsies performed during her treatment for cervical cancer. Her physicians did not seek her consent before using her tissue for research, nor did they receive any personal financial gain from the cell line.

The cell line did become extremely lucrative, however. Although it is difficult to precisely quantify the total revenue generated from the HeLa line, it is not unreasonable to assume that the line has contributed to hundreds of millions of dollars in downstream revenue. Hundreds of patents contain the word “hela” in their claims, and genetically modified versions of the line currently sell for as much as \$10,000.

For many, it seems an injustice that the Lacks family never received any financial benefits from the HeLa line, especially given that they lived in poverty, unable to pay even for their own medical care. Christoph Lengauer, a cancer drug developer and former Hopkins faculty member, articulated this sense of inequity when he reportedly told Lacks’s daughter that he thought Hopkins had “screwed up” by not sharing some of the proceeds from the HeLa cell line with the Lacks family (1). Although this sentiment resonates with a sense of fairness for many people, it requires critical examination before becoming accepted as precedent regarding payments to patients.

We recently had an opportunity to consider issues surrounding sharing revenues with patients who provide tissue for research when a young man (we will call him DF) was treated at Dana-Farber Cancer Institute for a



rare metastatic malignancy. Shortly before he died, he was admitted to the hospital with increasing shortness of breath, requiring placement of a pleural drainage catheter. With his knowledge and permission, the physician-investigators obtained discarded fluid from the catheter to obtain and isolate tumor cells. The cells were processed into a cell line that holds promise for basic science research and the development of therapeutics. The line may result in a revenue stream for the medical center, as well as personal income for the physician-investigators.

After the patient died, the physician-investigators who cared for him were motivated to see that his family received some financial benefit from his contribution. They sought advice from the Research Ethics Consultation Service at the Harvard Clinical and Translational Science Center, on which we serve.

Property rights in human tissue

If patients own their tissues, even after removal from their bodies, then it follows that they have the right to demand payment when a profitable discovery derives from them. One of the earliest cases addressing this question was *Moore v. Regents of the University of California* (2). John Moore had his spleen removed as part of his treatment for hairy cell leukemia. Several years later, he initiated a lawsuit after learning that his physician at the University of California, Los Angeles, had developed a lucrative

The intuition that tissue donors are owed financial compensation is mistaken as a matter of policy and ethics.

cell line (MO) from this tissue; at the time Moore predicted a market value of around \$3 billion. In 1990, the California Supreme Court decided that Moore did not have a property interest in his removed cells, worrying that giving property rights to patients would “hinder research by restricting access to the necessary raw materials” and might “destroy the economic incentive to conduct important medical research.” Most other legal precedent supports the view that patients do not maintain a property interest

in discarded tissue (3).

Even if patients lack such property rights, there are many examples of individuals receiving financial compensation for donating tissue. A striking case was that of Ted Slavin, a man with hemophilia who developed extremely high antibody titers after contracting hepatitis B (4). When his physician informed him that his blood might be valuable to medical researchers, he was able to sell his serum for as much as \$10,000 per liter, providing himself with a source of income for the rest of his life.

Are the Moore or Slavin cases relevant to those of Lacks or DF? What are the salient features that determine whether patients should be paid for their tissue?

Investigators’ obligations to individuals from whom they seek tissue for research

There are three distinct obligations that an investigator who seeks access to tissue might have toward an individual whose tissues, upon removal from the body, might hold value for biomedical research (see the table). In addressing each of these obligations, it is necessary to distinguish between situations in which the tissue constitutes excess material that remains after an indicated clinical procedure and those in which obtaining the tissue imposes incremental inconvenience, burden, or risk.

Consent: Residual clinical tissues, such as those at issue in the case of DF, are obtained as a by-product of necessary care,

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GENETICS

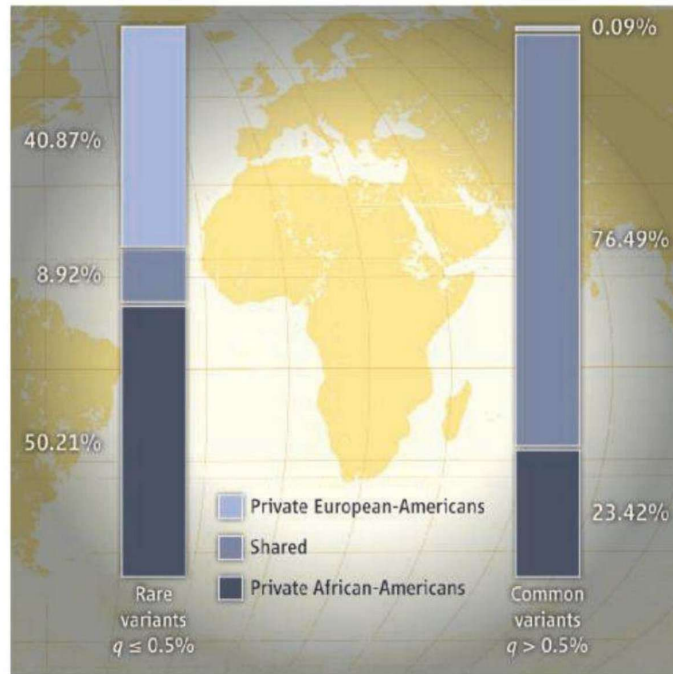
Human Genetic Variation, Shared and Private

Ferran Casals and Jaume Bertranpetit

The development of agriculture and livestock in the transition from the Paleolithic to the Neolithic era some 10,000 years ago heralded a demographic explosion in our species that is still ongoing today. Paradoxically, this rapid population growth, made possible by improved living conditions, may be responsible for an excess of damaging variants in our genome. Two papers in this issue, by Tennesen *et al.* (1) on page 64 and Nelson *et al.* (2) on page 100, report deep-resequencing analyses of all the human protein-coding genes (the exome) and of 202 genes that are putative drug targets in thousands of individuals, respectively. The studies show that most of the genetic variants occur at very low frequencies in human populations, which accumulate an excess of potentially harmful mutations.

Until recently, the snapshot of human genetic variation was mainly restricted to variants at frequencies above ~1 to 5%, because of technological and cost limitations. As such, medical and population genetics studies have been based on information derived from common variants called polymorphisms (and is the reason that studies have focused on single-nucleotide polymorphisms, or SNPs). Now, next-generation DNA sequencing technologies allow the analysis of thousands of samples and the characterization of less frequent genetic variation. This variation is younger than that studied previously and harbors information about more recent demographic events: There is an excess of rare variants (at a frequency $q \leq 0.5\%$) in relation to the existing models due to the explosive population growth of recent epochs (1–6).

Low-frequency variants are enriched for changes that alter protein sequence



Human genetic variation. Proportion of shared and unshared (private) variants between the African-American and the European-American populations [data from (1)].

and function (1–6) and, hence, are potentially involved in disease. The proportion of rare, functional nucleotide changes greatly exceeds the expected number predicted by classical population genetics analysis. This seems to be due to the inefficiency of natural selection in removing these variants because of the dramatic population growth in the last few thousand years (3, 4), which could lead to an important reduction of fitness in future societies (7).

This mutational burden in the population is also seen at the individual level. Each person carries some 35 nonsense substitutions that disrupt the formation of functional proteins. This number increases to 100 when all the loss-of-function mutations are considered, with 20 substitutions in a homozygous state (8), while 10 to 15% of the individuals are compound heterozygotes for nonsense variants in at least one gene. These figures raise the question of predicting the functional impact of DNA sequence variants not only at the local level of protein structure and function but also at the level of the organism.

Analyses of rare and common variants in the genome reveal another layer of human genetic variation within and among populations.

Not all loss-of-function variants will have the same phenotypic effect, with some better tolerated because of genetic and functional redundancy. Because the relationship between a mutation and its functional effect is not straightforward, more sophisticated predictive tools are needed that consider aspects such as the role and position in gene interaction networks and the final phenotypic effect given possible alternative pathways that may hide the deleterious or lethal effect of a mutation. Predictions will have to consider the effect on organisms, not only on protein structure.

The studies by Tennesen *et al.* and Nelson *et al.* sound two important warnings regarding the experimental design of association analyses for rare variants: Large samples sizes will be required (sometimes too large for actual studies), and replication across populations will be

limited, as most of the rare variants will be specific to each population. Genome-wide association studies, which include up to several million polymorphic positions throughout the genome, have provided insight into complex disorders by accounting for up to 10 to 20% of their genetic component (9). The rest of the heritability remains to be explained, and rare variants are among the alternative proposed factors (10, 11). Contingency tables comparing the allele frequency in control groups versus patients do not have enough statistical power because of the low frequency of rare variants, even for very large samples. Much effort is being invested in developing statistical tools to check for association of rare variants with complex disease, usually considering an aggregate effect of rare variants (12), which is of statistical interest but has poor biomedical interpretation. Thus, samples comprising thousands of individuals still have reduced statistical power even to detect large genetic effects, leading to the need for even bigger sample sizes (1).

Replication of the association across populations is crucial in genetic studies. In general, allele frequencies for common variants do not differ significantly across populations, especially within the same continent (13). But this picture becomes different for rare variants (14), as many will be population-specific. The sharing of rare variants is reduced to about 10 to 30% among populations in different continents (see the figure) and to 70 to 80% among populations in the same continent (1, 2). Consequently, those variants are more prone to show geographic stratification (due not to a predisposition to disease but to geographical differences), which may lead to false-positives (1, 15) and predicts a lack of replication across populations. Reduced allele sharing across populations points out the need for population-specific catalogs of variants, as well as careful collection of information about ancestry and correction for stratification. The genetic analysis of complex diseases through the comparison of cases and controls, which

has long been a widely used study design, may be approaching a conundrum that may not be solved just by increasing sample sizes and doing the most detailed type of analysis, the comparison of gene sequences.

Studies based on whole-genome sequences in thousands of individuals over the next few years should help to clarify the role of rare variants and their distribution in the genome and across human populations; such studies will represent an analytical and a computational challenge. But first, a better understanding of the functional impact of non-protein-coding variation, including regulatory regions, is needed as well as the development of tools to carefully functionally annotate genome variants. These steps would allow prioritizing of candidate variants and genes, both in the filtering approaches for rare Mendelian diseases and in the assignment of different weights in aggregate tests for complex disease studies and the interpretation of personalized genomic risk profiles. Ulti-

mately, we will need to go back to basic biology to link genome variation and phenotypes.

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PHYSICS

Two Atoms Announce Their Long-Distance Relationship

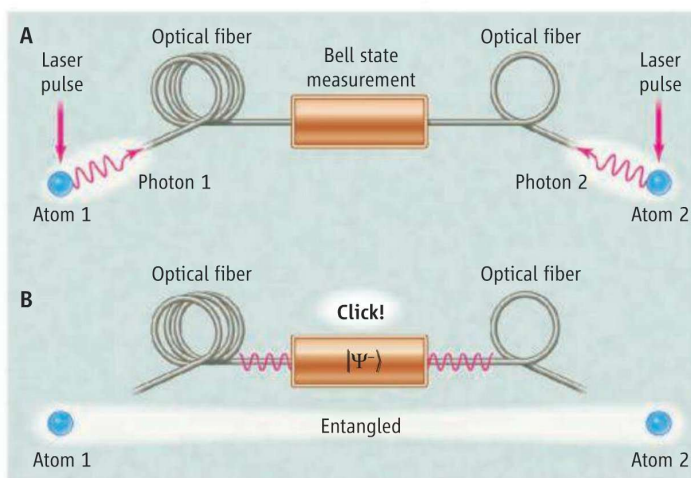
Jürgen Volz and Arno Rauschenbeutel

Entanglement is a counterintuitive property of quantum mechanics that puts it at odds with our classical view of the world. For example, an entangled state of two photons can be created such that each single photon is unpolarized—it will always pass through a polarization analyzer (e.g., a filter) with 50% probability no matter how the analyzer is oriented. However, once one of the two photons has passed through its analyzer, the second photon becomes polarized, and its orientation will then be orthogonal to the first. Such effects are not only interesting from a fundamental point of view: If the two entangled particles are shared between two distant parties, the perfect quantum correlations can be used to realize a so-called quantum channel over which quantum information can be transmitted. However, to exploit these schemes in long-distance quantum

communication, the generation of entanglement over macroscopic distances must be “heralded”—a separate signal must verify that the entangled state was created. On page 72 of this issue, Hofmann *et al.* (1) describe the accomplishment of this goal with two individual atoms held in laser traps and separated by a distance of 20 m.

Quantum-based communication and information-processing schemes promise

many advantages, such as the exponential speed-up of certain algorithms (2) or the possibility of detecting, with certainty, the presence of an eavesdropper in a communication channel (3). For this purpose, information is encoded in “quantum bits” (qubits)—the basic quantum information carriers that can, in contrast to classical bits, also exist in a superposition of the logical states “0” and “1”. Experiments making use of these effects



Telling on entangled atoms.

The experimental sequence developed by Hofmann *et al.* for “heralding” the entanglement of distant atoms is illustrated. (A) Each atom is excited by a short laser pulse and emits a photon that is entangled with the final atomic state. These photons are guided through optical fibers to a Bell state measurement setup. (B) Once an entangled two-photon Bell state like $|\Psi^-\rangle$ is detected, the atoms are also projected onto an entangled state. The success of the process is signaled by the “click” of the Bell state detector.

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are routinely achieved by using, for example, the polarization state of single photons (4). However, when sending single photons over distances of hundreds or thousands of kilometers (e.g., through telecommunication fibers), light absorption leads to photon loss, and the data rate eventually drops to impractical values.

Quantum-mechanical relay stations—so-called quantum repeaters (5)—can solve this problem by establishing a quantum channel between two parties in spite of optical losses. A necessary prerequisite for implementing a quantum repeater is the ability to faithfully entangle remote qubits. However, this process itself requires the exchange of single photons and is also impeded by transmission losses. For this reason, the entanglement procedure should ideally generate a signal that heralds the successful preparation of the entangled state. Using this feedback, the entanglement process can be repeated until it succeeds.

Such a heralded entanglement scheme has now been realized by Hofmann *et al.* with two macroscopically distant atoms. The experiment consists of two steps (see the figure). First, entanglement between a single atom and a single photon was created in two distant locations, and second, the two photons were projected onto an entangled state in a measurement. This second step swapped the entanglement (6) and resulted in the two atoms being entangled. In the present study, a single rubidium atom was trapped with optical tweezers (7) in two independent experimental setups at a distance of 20 m. Each atom was excited with a short laser pulse. After excitation, each atom decayed into one of two possible ground states. Depending on the state, the emitted photon had one of two orthogonal polarizations. However, as long as no measurement was carried out, it was not possible to know which decay occurred. Thus, the system was in a quantum superposition of both possibilities, i.e., in an entangled state between the state of the atom and the polarization of the emitted photon.

This atom-photon entanglement was then used to entangle the atoms. For this purpose, the photons were coupled into optical fibers, guiding them to a setup where a so-called Bell-state measurement was performed. This measurement projected the state of the two photons onto one out of four possible entangled states, or Bell states (6). Because they were independently emitted by the two atoms, the photons were initially not entangled, and the outcome of the measurement was random. However, the quantum correlations between the atoms and the photons resulted in the two atoms ending up

entangled once the photons were detected in a Bell state. Thus, the detection “click” in this Bell-state measurement constituted the heralding signal that confirmed that entanglement between the two remote atoms had been prepared.

Many experimental requirements had to be fulfilled to make both above steps work reliably. In particular, the authors took great care to ensure that the two photons were indistinguishable and that they arrived simultaneously at the measurement apparatus. Subnanosecond timing of the photon emission was essential, and the successful generation of entanglement showed that the atoms were well isolated from the environment in their laser traps. In addition, fluctuations of the photon polarization induced by the optical fibers had to be compensated to achieve the reported high degree of entanglement.

The demonstrated scheme deftly uses the advantageous properties of the different quantum systems involved—photons in optical fibers for transmitting the quantum information and atoms for information storage—

and can be readily scaled up to greater distances. The experiment represents an important milestone toward the implementation of practical long-distance quantum communication protocols. Moreover, the scalability in combination with the high purity of the atom-atom entanglement makes the system highly attractive for fundamental tests of quantum mechanics. In particular, it might enable a test of Bell's inequality (8) that closes all remaining loopholes (9) left by previous experiments and that gives a final answer on the nature of quantum physics.

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CELL BIOLOGY

A Mitochondrial Mystery, Solved

Ajit S. Divakaruni and Anne N. Murphy

A long-awaited transporter that moves pyruvate into mitochondria has been identified.

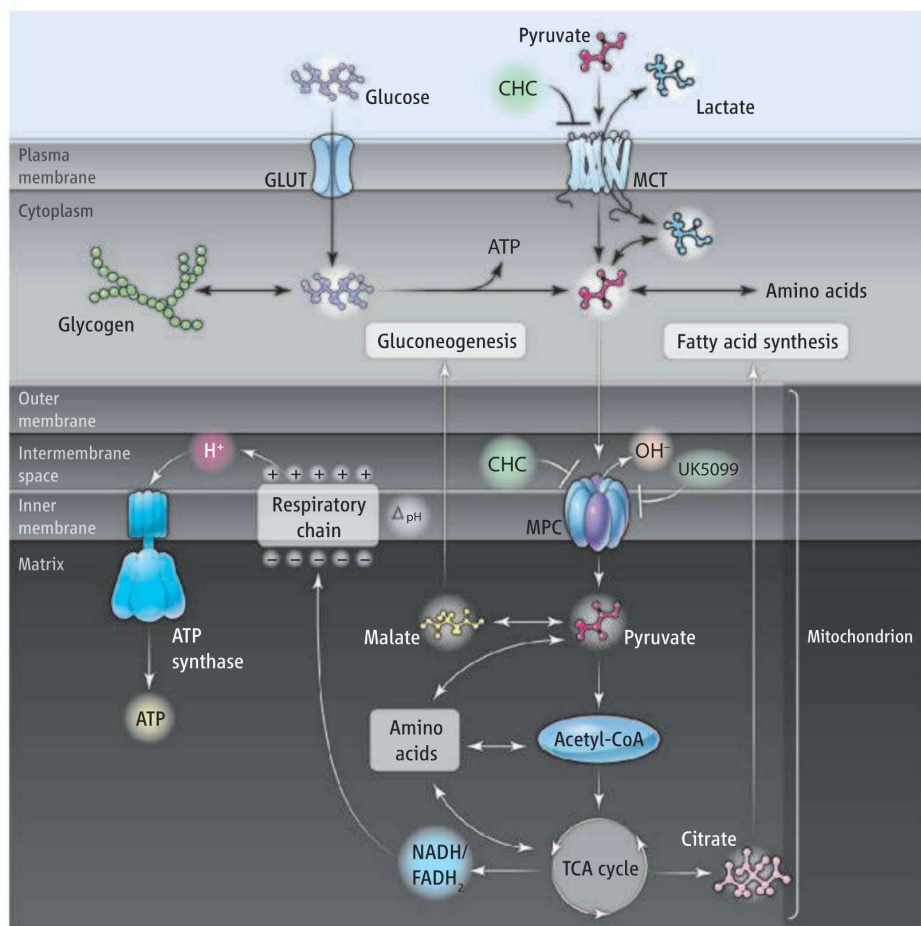
The three-carbon molecule pyruvate is a metabolic intermediate and a central hub for cellular energy metabolism. It lies at the junction of aerobic and anaerobic metabolism and is the precursor for biosynthetic pathways including glucose, lipid, and amino acid syntheses. Given the ubiquitous role of pyruvate in cellular bioenergetics (see the figure), its subcellular localization plays a critical role in its fate. The final product of pyruvate metabolism is fundamentally altered once it enters the mitochondrion. As such, the identity of the protein that transports pyruvate from the cytoplasm into mitochondria has been eagerly anticipated, and the wait has been nearly 40 years. Two papers in this issue, by Bricker *et al.* (1) on page 96 and Herzig *et al.* on page 93 (2), report the identification of a mitochondrial pyruvate carrier (MPC) responsible for this function—a momen-

tous development in the field of bioenergetics with profound implications for treating metabolic diseases.

Although pyruvate, a weak acid, can passively cross the mitochondrial inner membrane, extensive biochemical characterization demonstrated the specificity and kinetics of its uptake by mitochondria (3–8) (the outer membrane is not believed to provide a significant barrier as it is permeable to small solutes, including pyruvate). The discovery that thiol-reactive agents, such as α -cyano-4-hydroxycinnamate, inhibit pyruvate transport across both the mitochondrial and plasma membranes (3, 5) firmly established facilitated transport of pyruvate.

But the identity of the pyruvate carrier had been a long-standing mystery. Bricker *et al.* used an elegant combination of yeast genetics, metabolomics, and mutational analysis of cells from patients with defects in mitochondrial pyruvate metabolism to identify two MPC family members (MPC1 and MPC2), each ~15 kD in size, obligatory for mitochondrial pyruvate transport

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Paths of pyruvate. Pyruvate can either passively diffuse across the mitochondrial inner membrane or be transported by the mitochondrial pyruvate carrier (MPC); both processes are driven by ΔpH . MCT, monocarboxylate transporter; GLUT, glucose transporter; TCA, tricarboxylic acid; NADH, nicotinamide adenine dinucleotide, reduced; FADH_2 , flavin adenine dinucleotide, reduced; ATP, adenosine-5'-triphosphate; CHC, cinnamic acids.

in yeast, fruit flies, and mammals. MPC is likely a multimeric complex in the mitochondrial inner membrane, and deletions in genes encoding MPC components caused deficient carbohydrate metabolism in the fruit fly *Drosophila melanogaster* and in yeast; the latter also became auxotrophic for leucine. Metabolomics pinpointed MPC1 as facilitating the conversion of cytosolic pyruvate to mitochondrial acetyl-coenzyme A (CoA). Functional analysis with the high-affinity MPC inhibitor UK5099 (5) implicated MPC1 as a crucial mediator of pyruvate transport activity. Unequivocal proof, however, requires the reconstitution of inhibitor-sensitive transport activity in a system lacking MPC1 and MPC2.

Such proof is provided by Herzig *et al.* In addition to showing defective lipoic acid biosynthesis and impaired pyruvate uptake in yeast mitochondria lacking MPC paralogs, the authors demonstrated that MPC1 and MPC2 are sufficient to facilitate pyruvate uptake. When expressed in the lac-

tic acid bacteria *Lactococcus lactis*, which maintain a steady-state pH gradient (ΔpH) comparable to that in isolated mitochondria, neither MPC1 nor MPC2 showed appreciable transport activity. Coexpression of both proteins, however, was sufficient to reconstitute pyruvate uptake. The transport was inhibited by UK5099 and modulated by manipulation of ΔpH , two hallmark characteristics of the long-sought mitochondrial pyruvate carrier.

Why has the identity of the mitochondrial pyruvate transporter remained so elusive? Curiously, the components of this transporter may have been purified as early as 1981 (9), though subsequent efforts to identify the proteins proved difficult (10). Perhaps the greatest impediment was assumptions about its structure. Although the shared pharmacology of inhibiting pyruvate transport with α -cyano-4-hydroxycinnamate at the plasma and mitochondrial membranes hinted that the transporters might be related, transport at the plasma membrane was later

discovered to be facilitated by monocarboxylate transporters (MCTs, the SLC16 gene family, ~35 to 50 kD) with inhibitor and substrate specificities distinct from those of the mitochondrial pyruvate carrier (11). Another assumption was that the transporter should be a member of the mitochondrial carrier protein family (SLC25 gene family, ~28 to 34 kD) (12), which transports nucleotides, anions, and carboxylic acids involved in mitochondrial energy metabolism such as malate and citrate. As much of this family has yet to be functionally characterized, it seemed safe to propose that the mitochondrial pyruvate transporter would be a member.

The composition of the MPC complex is therefore extraordinary. The functional reconstitution experiments of Herzig *et al.* showed that neither MPC1 nor MPC2 in isolation can function as a monomer (unlike mitochondrial carrier proteins), but transport requires both. Moreover, immunoprecipitation and native gel electrophoresis conducted by Bricker *et al.* suggest that the MPC is a large heterocomplex, potentially with unequal stoichiometry between the two proteins. Such a structure is a fundamentally different paradigm for mitochondrial substrate transport and studies of its regulation could prove fascinating.

Given that the mitochondrial pyruvate transporter regulates a crucial branch point in cellular metabolism, its identification has enormous potential to treat human disease. One aspect is highlighted by Bricker *et al.*, who link mutations in MPC1 to families with a devastating multisystem genetic disease caused by impaired mitochondrial pyruvate oxidation. Sequencing of the *MPC1* and *MPC2* genes in the diagnosis of mitochondrial disorders may help tailor appropriate choices of dietary and therapeutic intervention for these patients.

More broadly, knowledge of the components of a pyruvate transporter may facilitate emerging drug discovery efforts targeted to mitochondria (13). Agents that enhance the rate of mitochondrial pyruvate uptake might reverse the Warburg effect, a hallmark of cancer in which tumor cells rely heavily on glycolytic metabolism despite adequate oxygen supply. Indeed, enhancing the rate of mitochondrial pyruvate oxidation with dichloroacetate (DCA) is being clinically evaluated for the treatment of cancer (14).

Although a systematic review of DCA as a treatment for mitochondrial disorders has not indicated widespread success (15), enhanced mitochondrial pyruvate transport and oxidation could prove effective for the

subset of patients for whom deficient pyruvate uptake and oxidation are causative of disease. Furthermore, the extent to which pyruvate transport controls the rate of oxidation has perhaps been underestimated in some tissues (9), so targeting the MPC may provide avenues for treatment where DCA has failed. Identification of the MPC also has implications for treating other pathologies, including heart failure, ischemia/reperfusion injury, and type 2 diabetes. Modulation of pyruvate transport could potentiate meta-

bolic flexibility and respiratory capacity that might be beneficial to treating these diseases.

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ASTRONOMY

Brown-Dwarf Origins

Shantanu Basu

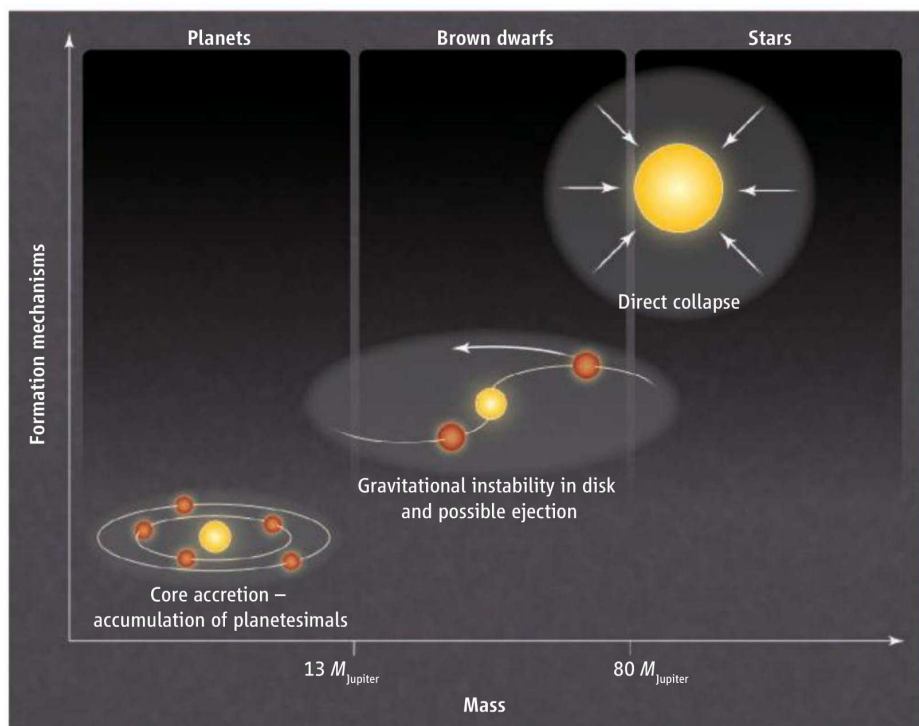
Brown dwarfs occupy an intermediate mass range, with too low a mass to initiate hydrogen fusion and become a star and too much mass to be called a planet (see the figure). Their existence was inferred theoretically nearly 50 years ago (1, 2), when it was realized that objects of mass less than about 0.08 solar mass ($M_{\odot}$) would initially contract due to gravity but then settle into a stable equilibrium due to quantum mechanical effects, before they became hot and dense enough to initiate hydrogen fusion. The first observational detections of a brown dwarf came in 1995 (3, 4), and there are now several hundred identified from infrared surveys (5, 6). The origin of brown dwarfs is an important astrophysical mystery because its solution can also determine how a broad range of objects, from high- to low-mass stars to planets, are formed and gain their masses. On page 69 of this issue, André *et al.* (7) report high-resolution observations with the Institut de Radio-astronomie Millimétrique Plateau de Bure Interferometer of the cloud core Oph B-11 in the Ophiuchus molecular cloud. Its mass is estimated to lie in the range ~ 0.02 to $0.03 M_{\odot}$, so that even a 100% efficient collapse into a central object would yield a brown dwarf.

A fundamental question in star and brown-dwarf formation theory is how the final mass is determined. Interstellar molecular clouds are the birthplaces of stars and brown dwarfs, and the vast majority of stars that form have masses within a few tenths of $M_{\odot}$, just above the hydrogen-burning limit. This is despite the minimum mass for gravitational collapse (the Jeans mass) being typically a few $M_{\odot}$, as is the

typical mass of dense cores observed in these clouds. Some inefficiency of infall to form central stars is expected due to the presence of powerful gas outflows that emanate from near a protostar and clear large cavities in the

Observations reveal the possible formation of a brown dwarf through the collapse of an interstellar cloud fragment.

surrounding medium (8). The requirement of much smaller masses for the final object than that of the Jeans mass has led to the proposal of continuing fragmentation of cores of mass $\sim M_{\odot}$. In the fragmentation scenario, free-float-



Stars, brown dwarfs, and planets. Stars are well defined as objects with enough mass to initiate hydrogen fusion in their cores ($M > 0.08 M_{\odot}$). They can form through direct collapse of molecular cloud cores, and the existence of many binary and multiple star systems implies that disk fragmentation can also form secondary stars. Planets are at the low-mass end of the spectrum and are thought to form only in disks, through coagulation of planetesimals or disk fragmentation. Brown dwarfs occupy a middle ground in mass, but the mass boundary with planets is subject to debate. A theoretical mass-based criterion places the boundary at about $13 M_{\text{Jupiter}}$ because a brief phase of deuterium fusion occurs at that minimum mass. An alternative criterion based on formation mechanisms may reveal that the boundaries between planets, brown dwarfs, and stars are not as rigid as the mass-based ones. Two separate mechanisms may be at work in each category. André *et al.* (7) present the first evidence for formation of a brown dwarf through direct collapse.

ing brown dwarfs are considered to be objects that formed in a multiple system but then got ejected from the system through many-body interactions (9). Ejections in many-body systems are a regular outcome of Newton's theory of gravity, with the lowest-mass members of a many-body system the most likely to be ejected. The ejection model has been challenged (10), however, due to a lack of observational evidence for fast-moving ejections. An alternative model is that molecular clouds can induce masses in the substellar range to collapse directly. The effect of turbulence in molecular clouds is invoked to create local high-pressure regions and induce core masses down to substellar values (11).

André *et al.* (7) use interferometer data to place an upper limit of 460 astronomical units on the radius of Oph B-11, with no evidence for an extended tail of emission that can be associated with this density peak and carry additional mass. The flux density of emission is used along with models for dust emission and a distance estimate to yield the total mass ~ 0.02 to $0.03 M_{\odot}$, with uncertainties not considered sufficient to push the actual mass into the stellar regime. Narrow spectral line widths imply that the gas is gravitationally bound. However, the surrounding medium has a large column density of gas and could add a substantial amount of mass if it accretes

onto a forming protostar in Oph B-11. André *et al.* use the classical Bondi-Hoyle accretion theory, applicable if the surrounding gas is not part of a self-gravitating system centered on Oph B-11, to estimate that the mass can only increase by $\sim 20\%$ in the typical time available for star formation in Oph B-11. The velocity of surrounding gas traced by spectral lines of N_2H^+ and DCO^+ reveals some evidence for supersonic interacting flows, which is consistent with the idea of very low-mass cores being formed by shocks.

Further interferometric measurements of individual sources will be necessary to determine whether star-forming regions contain an appreciable number of pre-brown dwarfs, gravitationally bound and distinct from their surroundings. At the same time, deep near-infrared surveys continue to detect finished brown dwarfs and establish their relative number to stars (12, 13). Can both mechanisms of low mass core collapse and ejection be at play? The turbulent theory of pre-brown-dwarf formation (14) predicts that they come from the low-mass tail of a core mass spectrum, so that they should be rare in comparison to stars. On the other hand, a recent hybrid ejection model (15) shows that ejections can occur soon after the formation of a circumstellar disk, with ejection of gaseous pre-brown dwarfs before they have fully

collapsed. These are accompanied by their own minidisks and also avoid the problem of high ejection speeds implied in the standard ejection models. In the ejection scenario, cores of larger masses can also give birth to free-floating brown dwarfs, so there is potential for greater numbers than in a direct-collapse model. Ultimately, the observed fraction of brown dwarfs, as well as the detection of more pre-brown dwarfs, is crucial to determine whether or not direct collapse is the main channel for their production.

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BIOCHEMISTRY

A Lipid Linchpin for Wnt-Fz Docking

Mariann Bienz¹ and Xi He²

This year marks the 30th anniversary of the discovery of Wnt1 (1), the founding member of a family of conserved secreted factors that define signaling pathways with pivotal roles in animal development, tissue homeostasis, and numerous diseases, including cancer (2, 3). Much later, the transmembrane proteins of the Frizzled (Fz) family were identified as the Wnt receptors (4). Wnt proteins proved extremely challenging to purify as active proteins; they require a lipid modification for activity (5), rendering them hydrophobic and insoluble in the absence of detergent. The resulting lack of structural data

has hampered mechanistic progress. The structure of the Wnt-Fz complex reported by Janda *et al.* on page 59 of this issue (6) provides important insights into how Wnt ligands interact with their receptors.

The authors used *Drosophila* S2 cells to coexpress near-full-length *Xenopus* Wnt8 and the extracellular cysteine-rich domain of mouse Frizzled 8 (Fz8-CRD) with an Fc affinity tag, which allowed them to purify a fully modified Wnt8-Fz8-CRD complex. They solved the crystal structure (at 3.25 Å resolution), to discover that Wnt8 forms a previously unknown fold, composed of two subdomains linked through a small interaction patch that may allow for torsional flexibility. Each subdomain extends a long fingerlike structure to contact Fz8-CRD at opposite surfaces (sites 1 and 2), apparently “pinching” it without changing its compact globular fold (see the figure, panel A) (7).

The crystal structure of a Wnt-Frizzled complex uncovers an unusual interaction mode, with a lipid in the binding interface holding the complex together.

Site 1 is truly unusual because it has a lipid in its center (which makes the complex detergent-sensitive): This lipid forms a fingerlike extension from the N-terminal domain of Wnt8 and consists of a >14 -carbon lipid chain (see the figure, panel A) attached to a serine residue (S187) at its tip. The electron density for this lipid is consistent with palmitoleic acid, the modification of the corresponding serine residue (S209) in Wnt3a (8). This lipid is partially embedded in a cleft between two α helices and a loop of Fz8-CRD, forming a linchpin in the complex that is likely to be the primary force driving the Wnt-Fz interaction. Site 1 involves numerous interactions that are chemically conserved among Wnt and Fz family members, which provides a simple structural explanation for the broadly degenerate interaction patterns (9) that allow an individual Wnt protein to pair with multiple

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Fz molecules and vice versa. The lipid may also have additional roles, for example, in facilitating Wnt secretion and/or its trafficking in the extracellular space via interaction with other proteins (10).

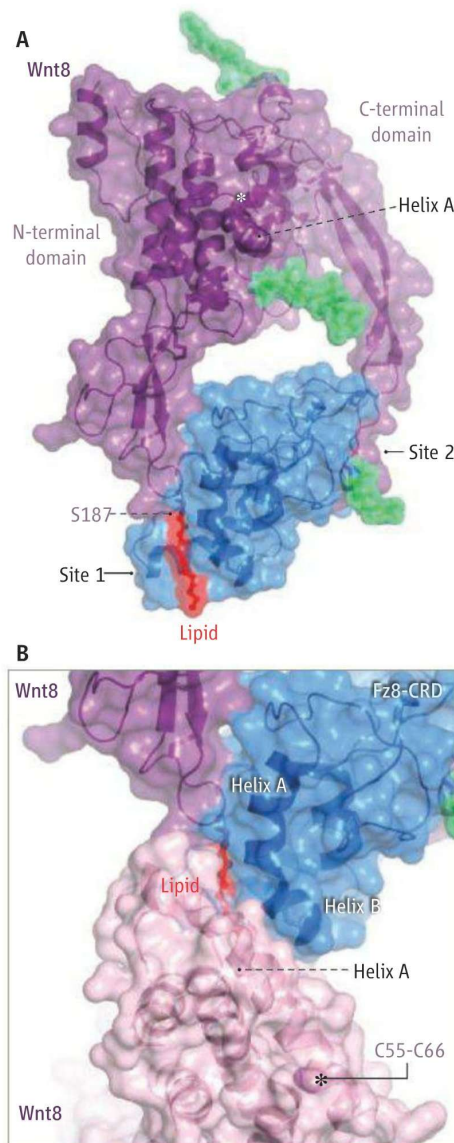
At site 2, a thin Wnt8 finger protrudes from the C-terminal domain of Wnt8 (see the figure, panel A). This finger consists of two long, twisted β strands and a connecting loop rigidified by several disulfide bonds, with the hydrophobic loop at the tip poking into a small depression of Fz8-CRD. At the center of this depression is a methionine residue (M149) that is conserved in a subset of Fz proteins but substituted by charged residues in others, suggesting that this residue might impose selectivity on Wnt-Fz pairings. Site 2 exhibits poor shape complementarity, but the authors validate this interaction by showing that the measured binding affinity of a water-soluble mini-Wnt8 for Fz8- and Fz5-CRD is in the low micromolar range, whereas it is undetectable for Fz4-CRD. Selective Wnt-Fz pairings may thus be attributable to site 2, but it is difficult to gauge how much this site contributes to the overall affinity between ligand and receptor, which appears to be determined mainly by the nonselective “lipid-in-groove” contacts at site 1.

Wnt ligands also directly interact with their co-receptors, such as LRP5/6, Ryk, and Ror2, which provide additional specificity and determine the downstream signaling events in the cell (11). Janda *et al.* identify a semiconserved surface patch on Wnt8 as a prime candidate for mediating its interaction with the extracellular domain of LRP6 (12).

The Wnt8-Fz8-CRD complex also bears glycan modifications (see the figure, panel A, green). These modifications are solvent-exposed and thus do not contribute to the structural integrity of the complex or to the Wnt-Fz interaction.

Somewhat unexpectedly, in light of previous results (5), the Wnt8-Fz8-CRD complex lacks a second lipid modification on cysteine 55 (C55), which forms an intramolecular disulfide bond with C66 (see the figure, asterisks). This bond is likely to be important for the structural integrity of the N-terminal α -helical domain fold and provides an explanation for the functional importance of the corresponding residue in Wnt3a (5). All Wnt ligands contain even numbers of cysteines (22 or 24), all of which are expected to form disulfide bonds, which are essential for the structural integrity of the Wnt fold as revealed by the structure of Wnt8.

The site 1 contact does not shield the lipid completely, leaving the complex aggregation-prone and insoluble in water. In the crys-



tal, shielding is provided by an alternative surface of Wnt8, mostly by its N-terminal α helix (helix A), which forms a lid covering up the lipid (see the figure, panel B). The authors cautiously term this “pseudo-site 3,” because there is currently no validation of its functional relevance. However, pseudo-site 3 exhibits the largest interface in the crystal and buries as much of the lipid face as site 1; it thus looks plausible structurally.

Were it to form, this interaction would create an asymmetric oligomer. Janda *et al.* report evidence for complex oligomerization from its behavior during gel filtration (6). Oligomerization of Fz-LRP6 receptor complexes after Wnt binding is crucial for signaling (13) and was proposed to trigger DIX-domain-mediated polymerization of Dishevelled (14), which enables it to assemble a signalosome at the plasma membrane through direct interaction with Fz (15). Functional validation of pseudo-site 3 is

Lipid at the interface. (A) The crystal structure of the Wnt8-Fz8-CRD complex reported by Janda *et al.* (shown here as a ribbon diagram superimposed on surface representation) contains two contact sites (sites 1 and 2). Asterisk, disulfide bond between C55 and C66, which stabilizes helix A; green, solvent-exposed glycan modifications. Note the central linchpin position of the lipid in site 1. (B) Close-up of site 1 after addition of a second Wnt8 molecule (light pink), which covers the solvent-exposed face of the lipid by forming a lid. Janda *et al.* call this third interaction “pseudo-site 3” because it awaits functional validation. The site is of interest because it creates asymmetric oligomerization of the Wnt-Fz receptor complex.

eagerly awaited, because this additional contact between Wnt and Frizzled could provide an elegant mechanism for how Wnt binding to its receptor activates downstream signaling via receptor oligomerization and consequent Dishevelled polymerization.

Janda *et al.*'s Wnt-Fz complex structure offers insight with unprecedented precision into how Wnt ligands interact with their Fz receptors, and this structure is a crucial step toward a better understanding of Wnt transmembrane signaling. It will empower more ambitious efforts to cocrystallize the whole Wnt receptor complex, including the entire serpentine Fz protein and its co-receptors and, ultimately, the Dishevelled signalosome. Such structural information will also facilitate rational drug design to enhance or suppress Wnt-receptor interactions for therapeutic purposes.

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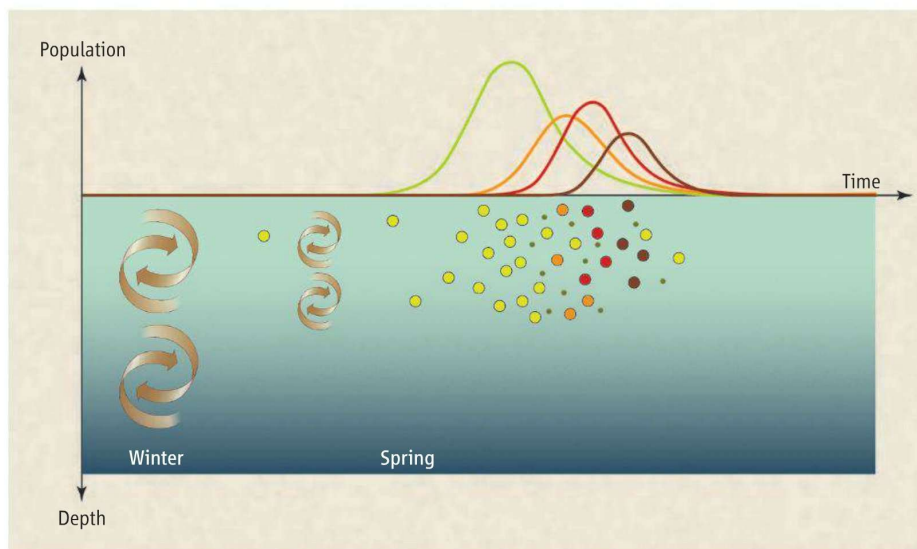
The Seasonal Smorgasbord of the Seas

Adrian Martin

The spring bloom of phytoplankton—an annual population explosion that propagates poleward across much of the open ocean and spills across the continental shelves—is a seasonal bounty for the marine ecosystem. As it wanes, its annual legacy is a flux of carbon out of the atmosphere as the organic material, containing newly fixed carbon, sinks. On page 54 of this issue, Mahadevan *et al.* (1) suggest that the bloom can be triggered by instabilities in surface currents that trap phytoplankton near the sunlit surface. In another study, Teeling *et al.* (2) recently suggested that the bloom itself may help to explain the “paradox of the plankton” (3); how can a seemingly homogeneous ocean sustain thousands of species?

The long-standing theory for what triggers the spring bloom is the critical depth hypothesis (4). Only the top ~100 m of the ocean receives sufficient light for phytoplankton reproduction, but growth often strips this layer of nutrients. According to the critical depth hypothesis, stirring by winter cooling and winds brings deeper nutrient-rich waters up to the surface, but this benefit is often outweighed by phytoplankton being in turn stirred down into deeper waters, where there is too little light to prosper. In spring, the base of this mixed layer shallows. This shallowing was thought to be mainly caused by seasonally increased heating leading to stratification of the water. Simultaneously, day length and light levels increase. At a “critical depth” of the mixed layer, the growth allowed by the average light level experienced by phytoplankton is balanced by the losses due to processes such as consumption by zooplankton and respiration. When the mixed layer shallows beyond this critical depth, the phytoplankton population grows exponentially, creating the bloom (see the figure).

Traditionally, stratification at a given location has been attributed to the local surface heat input or wind-induced mixing. Mahadevan *et al.* (1) bring recent breakthroughs in understanding surface ocean physical processes at scales of 1 to 10 km



What happens in a bloom. In spring, a combination of less mixing, reduced “grazing” (the consumption of phytoplankton by zooplankton), and increased light allows phytoplankton (green) to grow exponentially in a bloom. Mahadevan *et al.* argue that instabilities in surface currents provide the necessary reduction in mixing. Teeling *et al.* demonstrate that as the bloom wanes, through mortality and grazing, the organic material released (green dots) provides a range of resources that can be exploited as ecological niches by a variety of bacteria (orange, red, brown).

to bear on the issue. Surface waters can stratify much earlier than by heating alone as a result of instabilities in surface currents that cause lighter water to slide over denser water (5). Focusing on the open North Atlantic southwest of Iceland, Mahadevan *et al.* argue that this process can advance bloom onset by 3 to 4 weeks. The exact conditions under which this phenomenon occurs remain uncertain, hindering estimates of global impact.

Previous observations showing blooms beginning before stratification have led others to revisit the critical depth hypothesis. Behrenfeld has suggested that the bloom is triggered not by an increase in the rate at which phytoplankton reproduce but by a decrease in the rate at which zooplankton consume, or “graze,” them (6). In contrast, Taylor and Ferrari have argued that mixing can decrease before the tell-tale signature of stratification appears, such that stratification is not a reliable proxy for bloom initiation (7). With a variety of explanations, the challenge is to determine the balance of drivers in dictating the start of the bloom.

A globally dominant single influence is unlikely. Both biological controls, such as grazing (6), and physical ones, such as intensity of mixing (7) and susceptibility of surface currents to instabilities (1), vary markedly from place to place.

Even if its drivers remain debated, the bloom itself may help to resolve one of marine ecology’s most enduring debates. The competitive exclusion principle (8) posits that the number of species should not exceed the number of resources: One species will inevitably have some advantage in acquiring a given resource and will ultimately outcompete the other species for it. If a species is excluded from all resources by competition, it will become extinct. For phytoplankton, it is difficult to see how species diversity is maintained if the only resources considered are light and the most abundant mineral nutrients, such as nitrate, phosphate, and silicate.

One possibility is that we are underestimating the number of resources. By shifting the focus to bacteria, another component of marine plankton for which the “paradox”

should apply, Teeling *et al.* (2) show that the range of available resources and adaptations to exploit them provides sufficient ecological niches in the shallow southeastern North Sea to allow a diverse range of bacteria to prosper in the wake of a phytoplankton bloom when there is abundant organic material (see the figure).

These considerations provide an interesting counterpoint to alternative explanations, which question whether a plankton ecosystem ever achieves equilibrium—an assumption underlying the competitive exclusion principle. The system may be kept out of equilibrium by fluctuations, either in the environment (9, 10) or arising from nonlinear interactions of its component species (11, 12). Either way, conditions may never be the same long enough to allow one species to outcompete others for the limited resources and to drive them to extinction.

These explanations harmonize with that of Teeling *et al.*, who argue that the rapidly changing environment associated with the bloom provides the niches exploited by the various bacteria. Spatial heterogeneity of the environment—evident in the strong spatial gradients in figure S1 of Teeling *et al.*—may provide further niches. Modeling (13)

suggests that the dynamics of surface currents, such as those studied by Mahadevan *et al.*, can lead to adjacent but segregated patches of water with differing phytoplankton communities.

The growing debate over the causes and consequences of the spring bloom illustrates a deepening understanding of how the marine ecosystem operates. Substantial practical challenges lie ahead for furthering research. Multiyear time series are required to assess single-bloom studies (1, 2) more robustly and in a greater range of locations. Teeling *et al.* use an impressive portfolio of observational methods, but it is not clear how the study could be reproduced at locations farther from land, where sampling at the subweekly frequency necessary to resolve the rapid changes in populations during the short-lived bloom is technically extremely challenging. To test further the hypotheses for bloom initiation requires observations including turbulent mixing rates, zooplankton grazing rates, and detailed three-dimensional maps of surface circulations, at a time when weather conditions are most inhospitable to research ships.

Mahadevan *et al.* use modeling to augment their observations. Such a multidis-

ciplinary approach is promising, but additional focused observations are needed to configure localized process models, such as that in (1), frame hypotheses, and test model extrapolations to larger areas and different locations. Autonomous vehicles and sensors will play a key role, providing data throughout the year regardless of the weather. But for some time yet, scientists will continue to depend on and be constrained by short-period, ship-based sampling for many biological measurements, such as grazing rates.

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CANCER

Haploinsufficient Gene Selection in Cancer

Chris D. Greenman

Cancer is caused when normal signals regulating cell growth are disrupted by mutations in cancer genes, which are now known to number in the hundreds (1). These “driver” mutations are generally localized, only affecting the implicated cancer genes to produce a clear functional response that drives tumorigenesis. Cancer genomes, however, harbor thousands of mutations (2–4), only a handful of which affect cancer genes. The majority are “passenger” mutations that are likely to have little or no functional consequence. These mutations exhibit great diversity in character and some can affect large genomic regions containing many genes. This raises

the question of whether such large genomic assaults can be entirely passenger in nature. On page 104 of this issue, Solimini *et al.* (5) study one such common class of mutation known as hemizygous deletion and demonstrate that the cumulative response from the genes affected is sufficient to promote tumorigenesis.

Cancer genes broadly fall into two classes: oncogenes and tumor suppressor genes. Oncogenes promote cancer growth when sufficient signal is achieved. This often arises from single-nucleotide mutation, but also from increased gene dosage via copy number alterations, such as with the transcription factor MYC (6), or fusions of two genes to form novel transcripts, such as the BCR-ABL translocation (7).

Tumor suppressor genes (TSGs), as their name suggests, inhibit tumor development; if both copies of the gene receive inacti-

Cell proliferation experiments suggest that cancer can gain an advantage by partially inactivating several genes simultaneously with single deletion events.

ating mutations, the suppressive action is switched off and tumor growth is promoted. This is the original two-hit hypothesis of Knudson (8), where statistical inference was applied to incidence patterns of inherited and sporadic forms of retinoblastoma to show that two mutations are necessary to switch off the gene responsible (*RB1*) and initiate the disease.

In general, these inactivating mutations can take a variety of forms, including nonsense mutations where the resulting protein sequence is truncated prematurely, single-nucleotide mutations at functionally important sites, and large-scale deletions excising entire genes from the chromosome (*RB1* exhibits all types). When this latter class of mutation occurs on both copies, we have a homozygous deletion (see the figure).

By performing experiments to detect regions of recurrently absent DNA in can-

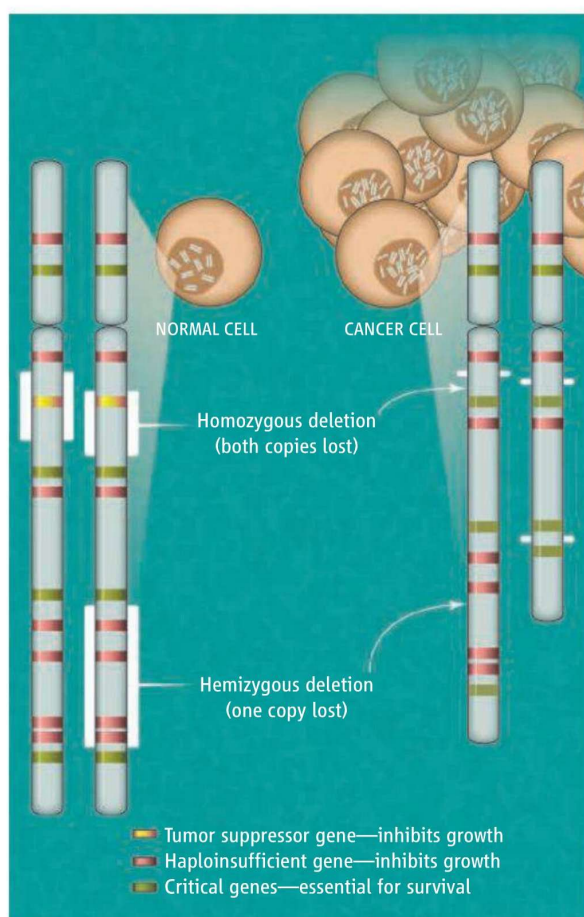
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cer samples, candidate TSGs can be identified and studied (9, 10). These experiments also highlight portions of the genome where only one allele has been deleted, termed hemizygous deletion. These events are far more common than homozygous mutations, and they tend to have larger genomic footprints. One hypothesis dictates that these deletion clusters contain haploinsufficient genes, where a reduction from two copies to one results in a phenotypic reduction in functional competence. If these genes have tumor-suppressive effects, the reduction to one copy may prove beneficial during tumorigenesis. The advantage gained may be small, but the large genomic footprint occupied by a single hemizygous deletion in a cancer genome has the potential to hit several haploinsufficient genes in one go, and the cumulative effect may be sufficient to provide a substantial selective advantage to an emergent cancer clone.

The tendency to delete one rather than both copies may arise because critical genes nearby cannot be homozygously deleted, but also, by preserving a single copy, the changes go unnoticed by the protective machinery of the cell (which might otherwise induce fail-safe mechanisms). For example, there is evidence (11) that haploinsufficient loss of *PTEN* can provide tumorigenic advantage while avoiding senescent signals of p53 that complete loss would induce.

Solimini *et al.* have taken a detailed look into these recurrent regions of hemizygous deletion, exploring several possible explanations for the clustering. By performing cell proliferation assays on telomerase-immortalized human mammary epithelial cells, they have examined how cell inhibition or proliferation capability is modified when suppressing the activity of genes found in hemizygous deletion clusters. In particular, they demonstrate that these regions are significantly enriched for genes inhibiting growth and lack genes critical for cellular function, providing evidence for the widespread selection of haploinsufficient targets (see the figure).

Alternative explanations for the common occurrence of hemizygous deletion clusters in cancer are possible. First, the deletions could just be the product of the random nature of mutational processes, with hemizygosity preserving one copy



Deleting genes in cancer. Selective growth advantages can come from a homozygous deletion erasing both copies of a single growth-inhibiting tumor suppressor gene (yellow), as well as from a hemizygous deletion targeting a larger number of haploinsufficient tumor-suppressing genes (red) while simultaneously avoiding deletion of critical (green) genes.

of the genes essential to cellular survival. However, the significant positional clustering of these deletions as well as the bias for certain gene types (mentioned above) make this improbable. Second, certain fragile regions of the genome are prone to deletion (10) and have a greater tendency for hemizygous (and homozygous) deletion. However, only a small proportion of the clusters examined overlapped with known fragile sites, and they tended to lack homozygous deletions that are associated with fragility. Third, these regions could contain TSGs, with the remaining allele inactivated by point mutations or epigenetic modifications. There is precedent for this: Chromosome 17 often exhibits loss of heterozygosity, for example, a likely second hit to TSGs (such as *TP53*, *NF1*, and *MAP2K4*) that inhabit this chromosome. However, the lack of homozygous deletions in these regions further supports the haploinsufficiency model presented by Solimini *et al.*

Further reinforcing their results is a recent study using mouse models to show that the long arm of chromosome 8, which frequently exhibits hemizygous deletion in cancer, contains several haploinsufficient targets that provide a selective advantage to cancer (12).

The classical models of mutation describe localized processes affecting the function of single genes (or two genes with fusions). However, there is increasing evidence that the evolutionary processes underlying cancer can find ingenious tricks to derive multiple selective advantages from one mutational event. For example, the extensive homozygosity observed in some chromosomes can arise from chromosomal loss, providing a second hit to more than one TSG, and complex rearrangements such as chromothripsis can target multiple sites (13). The work of Solimini *et al.* presents another example whereby individual hemizygous deletions target numerous haploinsufficient targets simultaneously to derive a collective growth advantage.

This work also provides more evidence of the varied landscape traversed by selective forces fueling tumor development (14). In addition to commonly mutated genes such as *TP53* that provide a huge fitness advantage to an emergent cancer clone, there are probably numerous infrequently mutated targets that offer small but significant cumulative advantage. This paints a complex picture of oncogenesis, but one that needs interpretation if we are to better understand the evolution of this debilitating disease.

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Network Interventions

Thomas W. Valente

The term “network interventions” describes the process of using social network data to accelerate behavior change or improve organizational performance. In this Review, four strategies for network interventions are described, each of which has multiple tactical alternatives. Many of these tactics can incorporate different mathematical algorithms. Consequently, researchers have many intervention choices at their disposal. Selecting the appropriate network intervention depends on the availability and character of network data, perceived characteristics of the behavior, its existing prevalence, and the social context of the program.

The importance of social network influences on behaviors is well established, and the advantages of network approaches to understanding a wide variety of phenomena are clear (1–7). Recent research on social networks and networking has shown that people can be influenced by their social networks to adopt new practices that affect their personal lives (1). There is widespread recognition that behavior and organizational change programs should be implemented and/or delivered by members of the group undergoing the change; i.e., the peers (8, 9). This accumulated body of evidence indicates that social networks can be leveraged to accelerate behavior change, improve organizational efficiency, enhance social change, and improve dissemination and diffusion of innovations. The purpose of this Review is to assess what is known from prior research on network interventions, provide a framework for organizing such efforts, and suggest promising new areas for future research and application.

Network interventions are purposeful efforts to use social networks or social network data to generate social influence, accelerate behavior change, improve performance, and/or achieve desirable outcomes among individuals, communities, organizations, or populations. Several caveats regarding network interventions are warranted: First, network interventions are not agnostic or impartial, but depend on the goals and objectives that initiate the intervention. For example, when the goal is to disrupt disease transmission, the intervention will be based on different tactics than an intervention with the goal of accelerating the adoption of disease-prevention measures (10). Second, scientific theory regarding how individuals or organizations change or can be changed is critically important for

choosing the right type of network intervention and the correct mix of promotional elements and materials (for instance, how much training of change agents, what type of media to use, and so on). Third, the interventionist should not only use the network as a delivery vehicle but also be prepared to use network information to learn from the community and to better serve community needs.

Network interventions are based on the diffusion of innovations theory, which explains how new ideas and practices spread within and

the network occurs such that novel interactions between people (links in the network) are activated; and (iv) alteration, interventions that change the network. The interventions are listed in order of increasing complexity, though not necessarily according to their efficiency. Each strategy has multiple tactical alternatives. For example, many programs identify individuals to act as “champions.” Tactically, however, the individuals who are identified might be opinion leaders, or they might be bridges between groups. Further, for each tactic there may be multiple definitions of the concept. For example, leaders are often defined as individuals who are most central in the network, yet there are at least a dozen different definitions and formulas used to measure centrality.

Figure 1 displays a hypothetical network used to illustrate the interventions, with solid circles designated as users of the behavior. This example, as well as many early network interventions, used relatively small networks such as classrooms ($n \approx 30$ people) or organizations ($n \approx 100$), but more recent studies have used online communities with thousands of members. The supplementary materials contain an R script file that can be used to replicate the calculations presented in this paper, as well as network graphs illustrating most of the interventions presented here.

Intervention Types

Individuals. In the most basic network intervention, network data are used to identify individuals to act as champions. The most frequent intervention of this type is the use of opinion leaders (13–15). At least 20 published studies have used nominations by members of the social network to identify leaders to promote behavior change. Most of the studies were randomized control trials designed to increase the uptake of evidence-based medical practices. In all cases, those who received the most nominations up to some threshold, the top 10 to 15%, were identified as leaders (nodes 28, 8, 13, 37, 19, and 6 in Fig. 1).

In addition to counting nominations, there are many mathematical algorithms available to identify central nodes based on different conceptions of centrality (16). For example, “centrality closeness” nodes (nodes 6, 37, 36, 13, and 35) can reach everyone else in fewer steps, on average, than other nodes. “Centrality betweenness” nodes occupy critical gate-keeping positions by most frequently lying on the shortest path connecting other nodes (numbers 6, 37, 36, 28, and 35). Other centrality measures (e.g., eigenvector, power, information, flow, etc.) might be

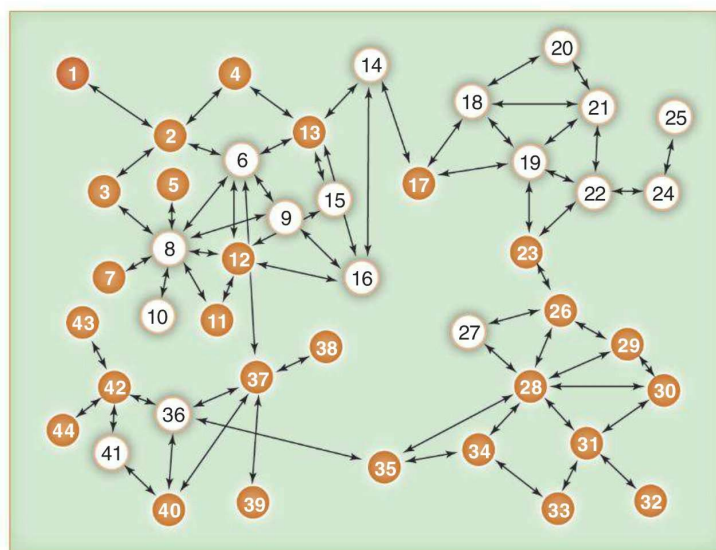


Fig. 1. Hypothetical network used to illustrate intervention techniques. Orange circles denote users (adopters); white circles indicate nonusers (nonadopters).

between communities (11, 12). Though diffusion and other mechanisms of social influence explain the process of change, they do not provide guidance on how to use that information to accelerate change. Here, we present four strategies that capitalize on network data to develop planned change programs. The criteria for the categories are: (i) identifying individuals (called “nodes” within the network) who are selected on the basis of some network property; (ii) segmentation, in which the intervention is directed toward groups of people; (iii) induction, in which excitation of

used depending on the goals and objectives of the program.

Borgatti suggested that the most critical nodes for behavior change programs can be identified by finding those that most optimally span the network (10). Furthermore, Borgatti observed that the most central nodes can sometimes be linked to the same people (6, 8); thus, using the number of links a node has to identify key players may not identify the best nodes to disseminate information or, if removed, fragment the network most efficiently. The KeyPlayer software (17) was developed to identify nodes that would be the best seeds for spreading a behavior (nodes 6, 22, 28, and 36) and those best for disrupting its spread (nodes 13, 23, 35, and 37).

Leaders may not always be the best change agents. Leaders have a vested interest in the status quo, whereas bridging individuals (who link non- or loosely connected groups) may be more amenable to change and may be in a better position to change others. For example, when diffusion between groups is expected to be difficult, bridging individuals may be more effective change agents (18, 19). Bridging individuals may be preferred as change agents when the behavior or policy is controversial or not likely to be well accepted initially. Bridging nodes can be identified as brokers (20) (nodes 8, 28, 6, 37, 19, and 13) who have many connections to people who are not directly connected or as bridges whose connections maximally increase network cohesion (18) (nodes 14, 37, 24, 6, and 35). In this case, node 37 has been identified as a leader and a bridge, indicating that this person is potentially in the best position to lead a change program because of his/her prominence and diversity.

Low-threshold change agents should be recruited when the researcher wants to create early momentum for the change and accelerate the time to reach a critical mass or tipping point. Low-threshold adopters are individuals willing to adopt a new idea earlier than their peers (21). If node 19 were to adopt an innovation, he/she would be doing so under the condition that two of his/her five contacts (nodes 17 and 23) were previous adopters. Exposure would thus be 40%, and, having adopted, the threshold would also be 40% (thresholds equal exposure at time of adoption). For person 20, his/her adoption at the same time would have been done with no adopters and, hence, an exposure and threshold of zero. In contrast, adoption by 27 and 36 would yield thresholds of 100%. Thus, nodes 19 and 20 have low thresholds (less than 50%), whereas 27 and 36 have high thresholds. Identifying low-threshold adopters as change agents would thus require some prior knowledge of behavioral adoption of a related innovation.

People on the margins of the community or organization may also be identified by change programs, because they are potentially excluded from services or the positive supports derived from community participation, not for their ability to persuade others. Individuals on the periphery

of a network learn about new ideas or practices later than their better-integrated peers and hence may suffer disadvantages from their exclusion. For example, in two classic studies of diffusion of innovation through social networks, social isolates were significantly less likely to adopt new farming practices or modern methods of contraception (12). In some cases, peripheral individuals may be important to identify, as they are often the source of new ideas and innovations because they have contacts with other communities and/or are free from the social pressure to conform.

Segmentation. In contrast to individual approaches in which certain individuals are recruited to be change proponents, segmentation approaches identify groups of people to change at the same time. For example, companies often introduce new procedures at separate locations sequentially rather than having all locations adopt the new procedures simultaneously. In some cases, behavior change is a group decision owing to the interdependent nature of the innovation or behavior change process (22). People often view themselves as members of a community of practice (23) with established norms and processes that can only change when the whole group changes (24). For example, a new workflow practice or technology standard may be difficult to adopt unless the entire group agrees to use the system at the same time. Communication technologies such as fax machines, texting, and social networking (for example, Facebook) increase in value as more users adopt the technology or standard (25, 26). Groups can either be mutually exclusive or “cliques,” which allow for overlapping group membership (27).

Group-detection algorithms create mutually exclusive groups and an overall index indicating how well the groups represent the overall network structure (28). The mutually exclusive groups defined from the data in Fig. 1 are illustrated in Fig. 2 and consist of (i) 3, 5, 7, 8, 9, 10, and 11; (ii) 1, 2, 4, 6, 12, 13, 14, 15, and 16; (iii) 17, 18, 19, 20, 21, 22, 23, 24, and 25; (iv) 26, 27, 28, 29, 30, 31, 32, 33, 34, and 35; and (v) 36, 37, 38, 39, 40, 41, 42, 43, and 44. Interventions can be delivered to the groups separately or sequentially.

A group structure that occurs in many inter- and intraorganizational networks is a core-periphery structure in which core members are densely connected to one another and peripheral members are connected to the core but not to each other (29). Mobilizing networks that have a core-periphery structure may be accomplished by focusing resources on the core members or by ensuring that the core members have sufficient resources or diversity to achieve network goals (30). For example, community coalitions are often composed of hundreds of organizations and/or individuals, yet the core working group may consist of no more than 20 organizations. Understanding who is part of this core and their distribution of assets is critical to coalition success. A study of a community coalition designed to improve health insurance coverage for children was deemed suc-

cessful because the core organizations spanned all of the services and functions needed to expand health care access (30).

Segmentation may also be designed to identify nodes that occupy the same roles in the organization or community (31, 32). For example, a new sales product might be communicated differently to the sales and technical teams. Similarly, in human service organizations, employees may be divided into positions such as line-service personnel, program managers, and program directors.

Induction. Induction interventions stimulate or force peer-to-peer interaction to create cascades in information/behavioral diffusion. Word-of-mouth (WOM) interventions stimulate interpersonal communication to persuade others to adopt the new behavior. Media marketing campaigns are often designed to generate buzz about their products, with the goal of increased sales (33), and frequently encourage users to recommend products to their friends and family (34). Often referred to as “going viral,” these interventions do not necessarily use network data, but they depend on the network for their effects. Research has shown that the success of WOM is a function of the network position of initial adopters and the incentives they have to recruit others (35, 36).

In respondent-driven sampling [(RDS), also known as “snowball methods”], individuals recruit others to participate in a study (for instance, a clinical trial) or receive an intervention (37, 38). In RDS, an initial set of people who are members of the community or population to be influenced are selected and identified as “seeds.” These seeds then recruit members of their social networks who subsequently encourage additional people to participate, and so on. Researchers can use coupons or cards as a means to track who recruited whom. Additionally, researchers must decide on the number of seeds to start with and how many others each seed can be expected to recruit. RDS is quite effective at connecting with hard-to-reach individuals who might not otherwise receive services. This is achieved by initiating recruitment with people who are members of this marginalized group. One of the initial studies applying RDS to the recruitment of injection drug users (IDUs) showed that an unbiased sample of IDUs could be recruited within three to five waves of recruitment (38). This tactic enables researchers to generalize their study results to a broader group of IDUs and ensures that interventions for IDUs reach everyone they are intended to reach. Adhering consistently to study procedures and protocols over these three to five waves of data collection can be challenging, however. One participant generated more than 100 recruits of varying ethnicity, gender, and place of residence. RDS differs from WOM in that RDS interventions require the seeds to recruit their closely-associated peers, whereas WOM interventions work by sparking interpersonal communication among any and all social ties.

Network outreach is similar to RDS, except that the network seeds recruit members of their

personal networks to participate in an intervention together, in which the behavior change messages can be delivered to the entire group. Network outreach is expected to be more effective than individual interventions because the motivations and lessons (such as preparation of healthy food) are delivered in a group context, and the group reinforces the positive behavior change (39, 40).

Leaders can be identified and groups matched or assigned to them, or the groups can be identified first and a leader selected afterward. Two randomized studies creating school-based substance abuse prevention programs using network analysis matched leaders to groups (41, 42). In both cases, the effects were dependent on contextual factors (who delivered the program and the social context of delivery) (41, 42). Figure 2 illustrates how leaders within groups are identified and expected to induce behavior change within their local networks (43, 44). The combination of different group-segmentation and leader-identification techniques provides a few dozen operational variations.

Alteration. Strategies one through three generally assume a static network (or ignore network dynamics). Many interventions deliberately alter the network to improve efficiency. Three different tactics might be considered: (i) adding/deleting nodes, (ii) adding/deleting links, or (iii) rewiring existing links. Adding nodes is an important and long-standing behavior change approach with outside change agents, expert consultants, and lay health advisors (LHAs) being deployed in many settings to accelerate behavior change. Many studies have used LHAs, who are community members trained in behavior change techniques (45). These LHAs fan out into the community, often going from door to door, to inform individuals and groups about health and other topics to promote behavior change. LHAs may sometimes work within their existing social networks or approach strangers at their homes, places of business, or in public areas. Politicians and advocacy groups often mount “get out the vote” campaigns consisting of door-to-door appeals, which have been shown to increase voter participation and diffuse to other household members (46). Support groups, such as Alcoholics Anonymous, are often used to add new people to a person’s network to facilitate behavior change. Node-addition interventions often create connections randomly, yet it is probably preferable to add nodes to the

network selectively on the basis of network position. New individuals should be added to a network to bridge disconnected or loosely connected groups (47).

Node-deletion interventions remove nodes that occupy critical positions in a network (48, 49). Nodes are then ranked on the degree to which their removal changes the network statistic. Node-deletion interventions have been embraced by antiterrorist agencies to degrade terrorist network organization (50). Removing critical nodes from sexual contact networks is an effective way for public health agencies to reduce disease spread and protect communities. In such cases, it is not always physical node removal but rather the use of protective behaviors (such as condom use) that inhibits transmission by the node. Node-deletion interventions change the focus of study from individual behavior to system dynamics in attempts to understand how communities or organizations respond to the removal or alteration of critical nodes. In the hypo-

thetical example in Fig. 1, the nodes that fragment the network most when removed are 6, 37, 36, 35, 8, 14, 17, 26, 23, and 28.

As with node deletion, network measures can be used to determine optimal connections to add or remove. Networks can be modified so that they have increased redundancy of the paths that connect individuals or how individuals connect to resources (51). For example, a 9-month study conducted in a global consulting agency revealed two distinct subgroups in the organization that did not communicate with one another (52). The intervention created extensive linkages between the two subgroups so that members throughout the organization knew the resources and assets available throughout the entire organization, not just within their own subgroup. Changing network structure is probably more difficult than using existing network structures (induction), because networks are often formed for a myriad of individual, relational, attitudinal, and environmental reasons.

Finally, networks can be rewired to increase efficiency or improve performance based on certain goals. For example, teachers often randomize classroom networks so that ability levels are randomly distributed in the network. As with node and link changes, the researcher can also maximize the network on one or several metrics. Watts has suggested that optimal networks are those with short average distances between nodes and a high degree of clustering (53). These small-world networks maximize bridging and bonding opportunities in the network. Finally, rewiring may be conducted to connect individuals with different attributes (e.g., a buddy system).

Selecting an Intervention

Selecting an appropriate network intervention depends on many factors, including the type and character of available network data, the type of behavior change being promoted, and the environmental or situational context. Network data can be derived from many sources, including archived communications (such as phone, e-mail, text messaging, and listserv postings), participant observations, published sources (such as corporate board membership), and survey data. Due to the plethora of network data sources, studies may vary considerably in their ability to

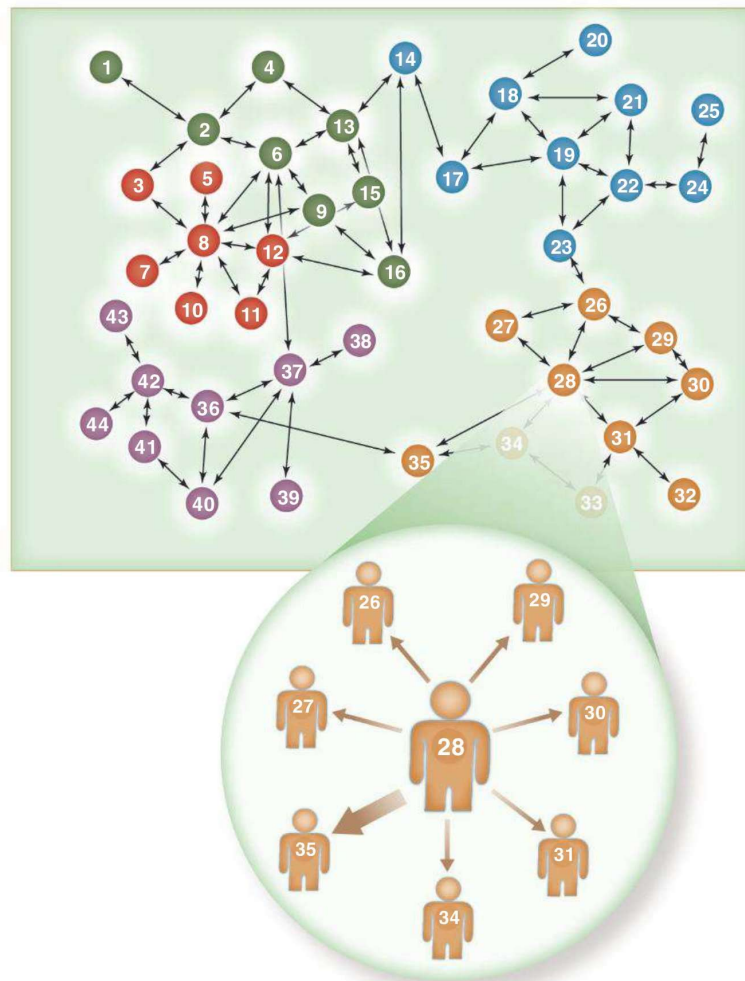


Fig. 2. Network segmentation, with each group represented by a distinct color (top). For induction, each group has a leader, and that leader influences the other group members (bottom). Node 28 can directly influence those immediately connected to him/her. Indirect influence will be required to reach those not directly connected to a leader. Different colors represent mutually exclusive groups.

assess the validity and reliability of the data. Indeed, an objective standard for what constitutes a social relation may or may not exist, depending on the social relation. For example, being friends with a person is somewhat subjectively defined, whereas having lunch together constitutes a known and certifiable relationship. Consequently, methods for assessing network validity and reliability are, by nature, incomplete. Evidence from survey research indicates that social networks can be measured validly and correspond to behavioral observations (54). From survey data, we know that people are more likely to recall strong ties as opposed to weak ones (55) and are reliable when using free recall or a list of names (56).

The classic medical innovation study conducted in the mid-1950s measured network connections among primary care physicians in four Illinois communities (57). The researchers asked physicians to name other physicians in their community with whom they discussed medical issues, turned to for advice, and considered as friends. The study concluded that social networks were associated with adoption of a new drug and that the advice and discussion networks were influential early in the process, whereas friendship mattered later. Advice networks identify people who are expert and credible sources of information and who usually have considerable technical knowledge about the idea or product (58). Discussion networks, in contrast, identify relations that are high in trust, mutual understanding, and interpersonal affect in which communication and persuasion flow easily. Discussion relations are mutual and close physically; advice relations are more likely to be asymmetric and distant. When barriers to adoption are technical or the innovation is complex, advice networks should be used for the intervention. In contrast, when barriers to adoption are primarily cultural, discussion networks may be more appropriate. Network interventions should measure different types of networks using the data for different strategies and tactics.

Geographic distance also plays a role: Smaller, local organizations will generally rely on trusted peers for information and not depend on geographically distant leaders, because local leaders provide advice that is more sensitive to local conditions and culture. Geographically distant leaders are still quite important, however, and they might have more technical knowledge than local leaders, which would make them valued sources of information. In addition to network type, overall network properties influence strategy selection. When network data indicate that the network is nonexistent, too fragmented, too centralized, or otherwise dysfunctional, there is a need for network change. The interventionist should use induction or alteration techniques to create a network amenable to change. Once the network is built or restructured, identification and segmentation tactics can be used to accelerate change. Network structure also matters. For example, a highly centralized network may profit from leader identification tactics, whereas a de-

centralized network will not gain much from using leaders, and instead the analyst must rely on segmentation or induction strategies.

Characteristics of the behavior being studied also affect intervention choice (11). A program designed to spread information of a readily accepted idea can rely on easily identified opinion leaders, whereas one that requires complex organizational and personal changes may need dynamic rewiring and/or matching of change agents.

Interdependent behaviors are those that increase in value as more people adopt them. For example, Facebook becomes more appealing as more of one's friends use this social networking site. Interdependent behaviors often have slow initial uptake because there are few advantages to being an early adopter. Thus, interdependent innovations benefit from segmentation strategies, induction matching, or rewiring so that the interdependence can be explicitly addressed.

Prevalence also affects intervention choice. At high levels of prevalence (greater than 75%), network interventions can be used to find individuals who have not yet adopted the behavior in question, perhaps due to their network position. At low prevalence (less than 15%), network interventions can identify whether early users are leaders and, thus, are well positioned to accelerate behavior spread or whether they are on the periphery and hence likely to be slowly imitated.

Perceived political support or acceptability of the new behavior can also influence intervention strategy. For example, in a study of public health officials in the 1960s, Becker (59) showed that opinion leaders were early adopters of measles immunization programs that were culturally compatible with the public health establishment, but these same leaders were later adopters of diabetes screening, which was perceived to be less compatible and riskier to adopt. In general, behaviors that are likely to be readily accepted are likely to be adopted early by leaders. Controversial change processes will need to invest in network feedback and employ segmentation tactics to reduce resistance. Programs perceived as being driven by a central authority are usually resisted.

Resistance to change and susceptibility to change are influenced by many non-network factors as well. It is critical for program staff to develop an extensive understanding of the variables that influence adoption. Well-articulated behavior change theory is critical to designing effective network interventions.

Existing evidence indicates that network interventions are quite effective. Leader-matching studies returned significant reductions in self-reported smoking and substance use rates compared those from with control groups (41, 42). Online peer-persuasion interventions have been shown to increase adoption of new products (34), and experiments manipulating social network exposures indicate that both peer persuasion and the network interconnectivity of those peers influence behavior change (60). Yet, the science of how networks can be used to accelerate behavior

change and improve organizational performance is still in its infancy.

Research is clearly needed to compare different network interventions to determine which are optimal under what circumstances. The challenge of distinguishing causation from correlation is exacerbated when making causal inferences from network associations. Linked individuals who exhibit the same behavior may do so because they influence one another, are influenced by others they have in common, or select one another to be friends based on the behaviors (i.e., two people who wish to smoke become friends to smoke together) (61). Some scientists have criticized causal inferences made from regression-type models (62), and statisticians have built elegant but complex models to address these challenges (63, 64). Although most interventions would benefit from such data in their evaluation, because network interventions are based on accelerating social influence, they require a deeper understanding of the social mechanisms driving behavior change. Further, such analyses may be critical in comparison of network interventions with non-network ones.

The options for network interventions have been dramatically enhanced by communication and information technologies that enable policymakers to identify critical nodes and network groups and to enhance diffusion within naturally occurring social networks. Electronic communications (social media, e-mail, text messaging, etc.) permit large-scale unobtrusive measures of social networks along with behavior change information. These technologies enable network interventions to go "to scale" and to be implemented beyond small-scale community groups or organizations.

However, these assets do not come without costs. Computer-mediated communications remove the richness of face-to-face interaction and were traditionally thought to be less effective for persuasion than interpersonal interaction. Moreover, many populations now live in media-saturated environments, making it hard to attend to any one message or medium. Research is needed on whether electronic networked interventions are more effective or efficacious than traditional face-to-face interaction (that is, does the increased reach compensate for the diminished effectiveness?).

Still, these electronic networks are often composed of friends (sometimes close friends), and much affective communication now occurs over electronic media. These socially mediated communications may be more relevant than mass media messages (65). Further, network interventions within computer-mediated environments can take advantage of data not previously available. For example, smart phone interventions can incorporate geographic location information that can be directly linked to the intervention. A network intervention promoting health screenings can include information on where and when screenings are available. Hence, the message encouraging screening comes from a friend and contains pertinent "point-of-sale" information

reducing barriers to behavior change. For example, a prototype system now in development uses wearable sensors to constantly monitor and communicate individual health data to the patients' health care providers (66).

The strategies and tactics presented in this Review can be implemented within an electronic environment. Individuals occupying critical nodes of various types (for instance, leaders or bridges) in electronic communications can be identified. Subgroups can also be identified and, using chat-room-like technologies, actually formed into groups with specific names; individuals within the groups can be encouraged to jointly adopt new behaviors and discuss their experiences. Many companies (e.g., Amazon, StubHub, etc.) already employ various induction strategies—for example, these companies may prompt individuals to share new purchase information with their Facebook friends or Twitter followers. In short, electronic communications already incorporate many types of network interventions, and many more can now be tested.

Empirical and theoretical work on network dynamics has shown that networks evolve in both predictable and unpredictable ways (63, 67, 68). Still, we currently have little understanding of how network evolution and dynamics may impact the effectiveness of these efforts. For example, does the network position of change agents differ if they act as sponsored change agents? It is the possibility of such a change that inhibits many leaders from participating in interventions. A leader who enjoys widespread power and control is unlikely to support interventions designed to change the status quo.

A further concern is that simply soliciting network information and/or feeding it back to community organizations can generate positive results. Network interviews prompt individuals to consider their interpersonal context, potentially making them aware of the contacts and ties they do and do not possess, which may affect their ability to achieve programmatic goals. Displaying network diagrams to agencies and organizations may thus prompt individuals to create or dissolve ties that alter network structure, regardless of any other programmatic activities.

The benefits of network interventions do not come without some risks. Organizational or community members may be reluctant to have their position in the network known by others. Some people may rightfully fear that their status or jobs may be jeopardized if the network data show them to be less (or more) important than expected. A person identified as an informal leader may be seen as threatening to management or demand a raise as a result.

The studies reviewed here indicate that networked interventions are more effective than non-network alternatives. To date, however, few of the many network intervention alternatives have been tested in laboratory or real-world settings, and it is unclear which network interventions work best under what conditions. There are many

strategic, tactical, and operational choices to be made when implementing a network intervention. Appropriate choices depend considerably on data availability, the behaviors under study, and the social context of the setting. Thus far, results suggest that these efforts will yield considerable scientific knowledge regarding the behavior, evolution, and malleability of socio-technical systems. By understanding how social networks can be used to improve learning, performance, and organizational outcomes, we can use the power of human interaction to improve the human condition.

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Supplementary Materials

www.sciencemag.org/cgi/content/full/337/6090/49/DC1
Supplementary Text
Figs. S1 to S22
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Computer Code and Data
10.1126/science.1217330

Eddy-Driven Stratification Initiates North Atlantic Spring Phytoplankton Blooms

Amala Mahadevan,¹ Eric D'Asaro,^{2*} Craig Lee,² Mary Jane Perry³

Springtime phytoplankton blooms photosynthetically fix carbon and export it from the surface ocean at globally important rates. These blooms are triggered by increased light exposure of the phytoplankton due to both seasonal light increase and the development of a near-surface vertical density gradient (stratification) that inhibits vertical mixing of the phytoplankton. Classically and in current climate models, that stratification is ascribed to a springtime warming of the sea surface. Here, using observations from the subpolar North Atlantic and a three-dimensional biophysical model, we show that the initial stratification and resulting bloom are instead caused by eddy-driven slumping of the basin-scale north-south density gradient, resulting in a patchy bloom beginning 20 to 30 days earlier than would occur by warming.

Springtime phytoplankton blooms in high- and mid-latitude oceans contribute substantially to global photosynthetic fixation and export of carbon from the surface ocean, with the North Atlantic alone accounting for 20% of net global CO₂ uptake (1). During winter, strong surface cooling and winds drive turbulent mixing that repeatedly carries phytoplankton to depths of several hundred meters, well below the lighted surface layer. Combined with low solar radiation, these factors limit winter phytoplankton growth. Springtime increases in solar radiation, coupled with weakening surface forcing and shallowing mixing depths (2, 3), cause the surface sunlit layer to retain phytoplankton for longer periods, promoting rapid growth that outcompetes grazing and leads to a phytoplankton bloom.

Vertical density stratification exerts a fundamental control on mixing depth, with strong stratification suppressing turbulence and reducing the depth of mixing. Previous studies, employing one-dimensional (1D) (vertical) models, attribute springtime increases in stratification to surface warming by solar radiation and air-sea heat exchange (4, 5). This study demonstrates a 3D process in which 1- to 10-km scale instabilities growing from horizontal density gradients give rise to eddies that generate vertical stratification, thus initiating a spring bloom earlier than expected from solar heating.

Deep wintertime mixed layers (MLs), though homogenized vertically, have a horizontal density gradient with heavier (colder) water toward the north and lighter (warmer) water southward (Fig. 1 and Fig. 2, B and D). In a nonrotating

system, the heavy northern waters would slide laterally under lighter southern waters, creating vertical stratification and releasing potential energy stored in the horizontal density gradient. However, Earth's rotation inhibits such movement (6), instead inducing flow normal to the horizontal density gradient that preserves both stored potential energy and deep MLs. Recent work has shown that the growth of instabilities (7) within the ML can tap this potential energy to develop energetic eddies, initially 1 to 10 km in horizontal extent and as deep as the ML. These eddies drive net horizontal transfer of lighter water above heavier water that can stratify the ML (8) on a time scale of weeks (Fig. 1 and movie S1). This process of ML eddy restratification competes with vertical mixing due to surface wind and cooling to set the density structure of the ML. High-resolution process-study models that resolve small ML eddies suggest that eddy-driven restratification is important for light-limited phytoplankton blooms in the Mediterranean (9, 10) and at high-latitude ocean fronts (11). Although parameterizations of ML eddy restratification developed from such studies (8) greatly improve the predictions of ML depth in climate models (12), direct observations of ML eddy restratifi-

cation (13) have been limited. This study provides observational evidence for springtime stratification by ML eddies and, with the use of a numerical model in the observational setting, shows that the process affects the timing and structure of the North Atlantic spring phytoplankton bloom.

Observations. In the spring of 2008, an intensive observational program (14) characterized the initiation and development of the spring phytoplankton bloom south of Iceland (Fig. 2A). North Atlantic Bloom 2008 (NAB08) measurements were collected in a patch-following reference frame defined by an autonomous, subsurface Lagrangian float (15). Four Seaglider robots—self-propelled, buoyancy-driven autonomous underwater vehicles—surveyed around the float. The array of autonomous instruments was deployed from R/V *Bjarni Saemundsson* on 4 April 2008 (yearday 95; yearday 1 = 1 January 2008), augmented by detailed measurements of physics, biology, and chemistry from R/V *Knorr* in May. Our analysis uses measurements of potential density from all seven platforms; chlorophyll concentration additionally includes Moderate Resolution Imaging Spectroradiometer (MODIS) satellite data (see supplementary materials and methods). Potential density profiles obtained from Argo floats that sampled this region between 2000 and 2009 provide a climatological picture.

In early April 2008, potential density ρ profiles (Fig. 3A) showed well-mixed layers 200 to 300 m deep, with ρ increasing toward the north (y direction) in both NAB08 measurements (Fig. 2B) and climatology (Fig. 2D). The lateral density gradient ρ_y was composed of a series of fronts with large ρ_y , interspersed with regions of more laterally uniform density (Fig. 2B). Vertical stratification increased dramatically during the measurement period, so that by late May the mixed layer was often only 10 m thick (Fig. 3B). Buoyancy frequency $N^2 \equiv -(g/\rho_0)\partial\rho/\partial z$ (where g is the acceleration due to gravity and ρ_0 is a reference density), a function of the vertical density gradient, provides a quantitative measure of stratification, which we averaged over shallow (0 to 100 m) and deep (100 to 300 m) ranges.

Springtime stratification develops in two distinct phases. Deep wintertime MLs initially

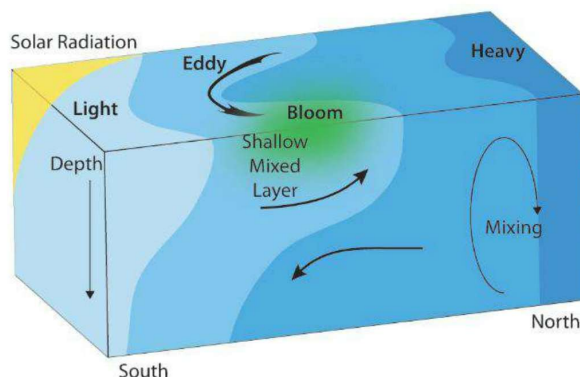


Fig. 1. In early spring, the deep, well-mixed layers created during the winter are denser to the north. Eddies move heavier water south and downward and lighter water north and upward, creating patches of shallow MLs that retain phytoplankton within the upper sunlit region and initiate the spring bloom. [Graphic by K. Mahadevan]

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progress from well mixed to linearly stratified across the upper 200 to 300 m (Fig. 3A, blue to orange profiles). Average N^2 in both the shallow (Fig. 3B, solid lines) and deep (dashed) layers is small in midwinter and increases concurrently to about $3 \times 10^{-6} \text{ s}^{-2}$ between yeardays 100 and 125. A strong storm produced the short-lived decrease in NAB08 stratification near yearday 120. In the second phase, stratification in the shallow layer increases sharply, diverging from the deep layer. Both the NAB08 data (Fig. 3B, red) and the climatological Argo data (Fig. 3B, green) show this pattern, albeit with somewhat different timings.

The observations suggest that ML eddies introduce the initial stratification across the entire depth of the wintertime mixed layer, whereas surface warming acts later and increases only shallow stratification. Estimates of air-sea heat flux Q [National Oceanography Centre (NOC) 1.1a climatology (16) (supplementary materials, section 1.8)] indicate that the atmosphere cooled the ocean until roughly yearday 120 (Fig. 3D). During this period, the temperature measured by the

float decreased by 0.4°C , a rate roughly consistent with the heat fluxes in Fig. 3D and measured ML depths (fig. S10). Thus, the first phase of stratification occurs during a period of surface cooling and entails a vertical density difference of 0.12 kg m^{-3} (Fig. 3A), which is the same as the lateral density change over $\sim 250 \text{ km}$ (Fig. 2B). The initial increase in stratification spans the upper 200 to 300 m and is produced by a layering of warm, salty water of southern origin atop colder, fresher waters originating from the north. These features are consistent with the formation of vertical stratification by eddy-driven slumping of the lateral gradient. Net surface heating starts on yearday ~ 120 , which is approximately when the second, surface-intensified phase of stratification begins. Thus, both the magnitude and timing of the heat flux and the horizontal and vertical structure of the upper ocean support the hypothesis that ML eddies drive the initial restratification.

As postulated by Sverdrup (17), initiation of the spring phytoplankton bloom occurs concurrently with the observed onset of stratifica-

tion. Chlorophyll concentration (a surrogate for phytoplankton biomass), which is measured by autonomous, ship-based, and MODIS satellite sensors, remained at low levels in midwinter, then increased rapidly between yeardays 110 and 130, marking the onset of the spring bloom (Fig. 3C). Diatoms dominated the bloom, which terminated (18, 19) due to exhaustion of silicate, an essential nutrient for diatoms. This study focuses on the initiation and evolution of this diatom bloom, up to the time of silicate limitation.

Theory. Competition between the stratifying effects of ML eddies and the destratifying effects of surface cooling modulated by wind forcing governs the development of stratification within the ML in winter. Lateral density gradients within the surface ML are unstable (7, 20) and generate ML eddies, which move parcels of heavy water southward and downward and parcels of light water north and up (Fig. 1). The overall effect of the eddies is to convert the horizontal density gradient into vertical stratification. Convective mixing, driven by surface

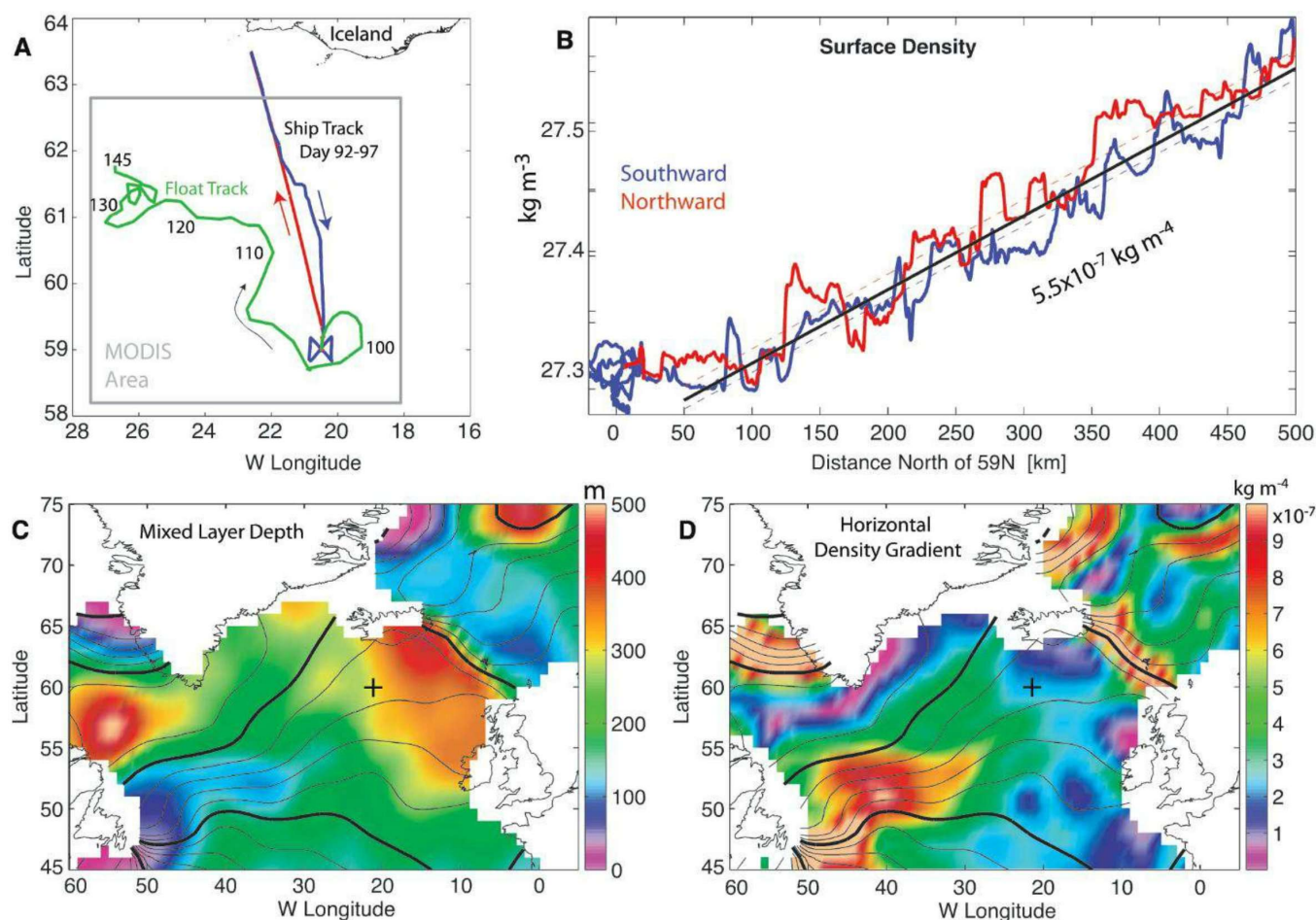


Fig. 2. (A) Experimental region showing the Lagrangian float track (green) labeled by yearday and the deployment cruise track (blue, southward; red, northward). The gray box demarcates the region of averaged MODIS satellite chlorophyll in Fig. 3C. (B) Surface density along the ship track, colored as in (A). Dashed lines give fits for inbound and outbound tracks; the solid black

line fits both. Slope differences are less than 2%. Maps of (C) ML depth and (D) magnitude of horizontal density gradient from 1997-to-2009 Argo data (35), smoothed with Gaussian scales of 2° latitude and 3° longitude, both with contours of density (contour interval of 0.1 kg m^{-3}). Black crosses denote the approximate experimental location.

cooling, counters the effects of ML eddies by homogenizing the water column and destroying vertical gradients. In the presence of Earth's rotation, winds induce a surface Ekman transport normal and to the right of the wind in the Northern Hemisphere. Thus, a wind along the density front in the direction of the frontal current ("down-front") drives heavy water over light, producing convective mixing similar to that driven by surface cooling (21). The balance between restrati-

fying mechanisms (for instance, ML eddies, solar heating, and up-front winds) and mixing (due to, for example, surface cooling and down-front winds) controls ML depth.

The stratifying buoyancy flux due to ML eddies (8) for a single front is proportional to $(b_y H)^2 / f$, where H is the ML depth, b_y is the initial meridional gradient of the buoyancy $b = -g(\rho - \rho_0)/\rho_0$ at a front, and f is the Coriolis frequency. The ratio of the buoyancy fluxes due to cooling (de-

stratifying) and ML eddies (stratifying) is estimated as $S = \frac{-g\alpha}{0.06\rho_0 C_p} \frac{Q}{b_y H^2}$, where Q , α , and C_p are the air-sea heat flux, thermal expansion coefficient, and specific heat capacity of seawater, respectively. We choose $b_y \sim -0.3 \times 10^{-7} \text{ s}^{-2}$, a value close to the density gradient along the tracks in Fig. 2B. With $H \sim 300 \text{ m}$, $C_p = 3988 \text{ J/kg/}^\circ\text{K}$, $f = 1.28 \times 10^{-4} \text{ s}^{-1}$, $\rho_0 = 1025 \text{ kg/m}^3$, and $\alpha = 1.6 \times 10^{-4} \text{ }^\circ\text{K}^{-1}$, we estimate that $S = 1$ would be achieved at $Q \sim -100 \text{ W/m}^2$. This implies that, in

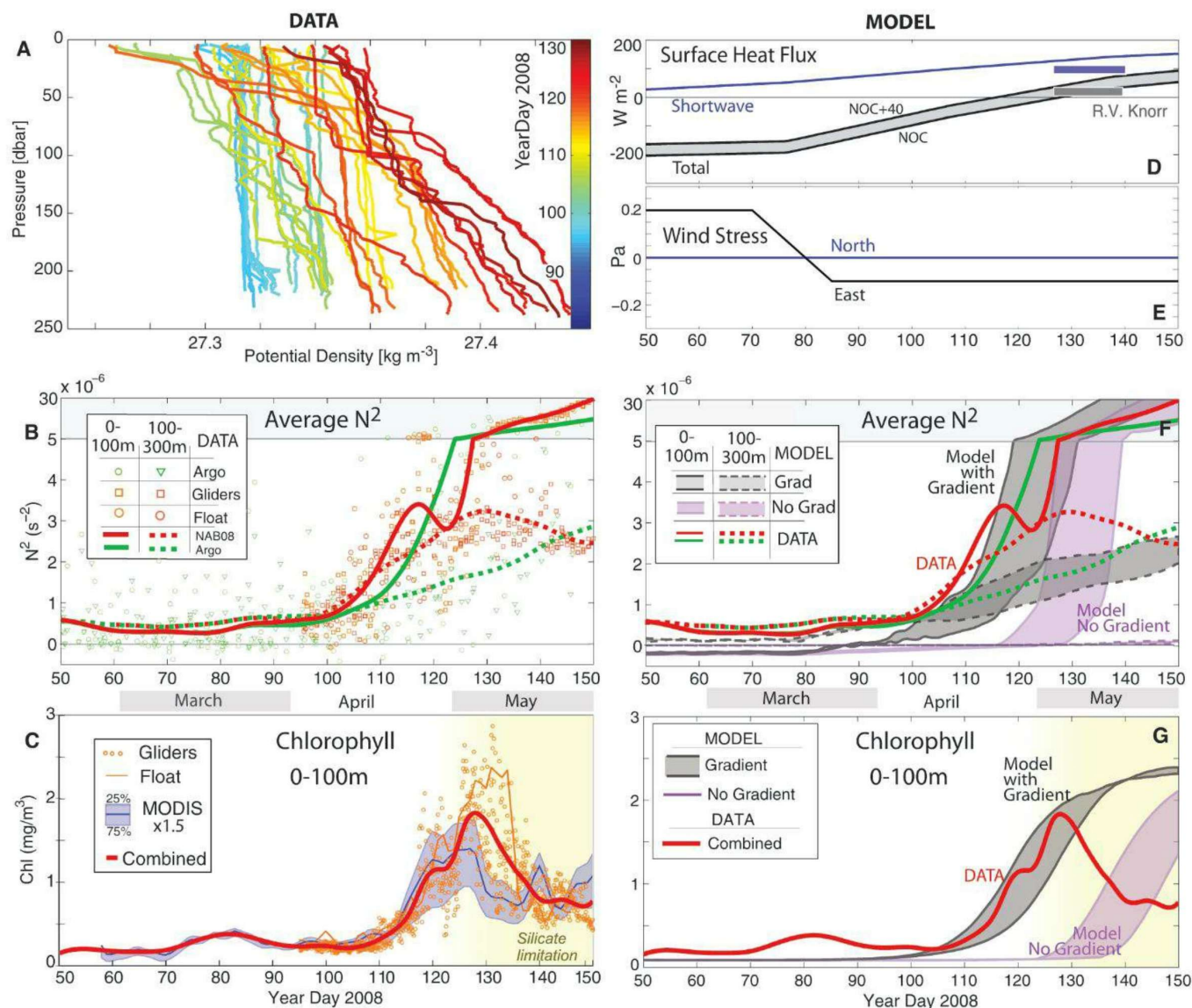


Fig. 3. Comparison of data (left column) and model (right column). (A) Evolution of vertical profiles of potential density at the float. Line color denotes time (yearday 2008). (B) Stratification, average value of N^2 , as measured by Argo floats (green), gliders (NAB08; red), and Lagrangian float (NAB08; orange) between 0 and 100 m and 100 and 300 m. Lines (solid, 0 to 100 m; dashed, 100 to 300 m) are smoothing spline fits to the Argo (green) and NAB08 (red) data. (C) Chlorophyll (Chl) 0 to 100 m from float (orange line), Seagliders (dots), and MODIS satellite (blue with 25 and 75 percentiles) from within the gray box of Fig. 2A, multiplied by 1.5 to calibrate to observations. Smoothing spline (heavy red line) is fit to all data. Yellow shading indicates the onset of silicate limitation in the data. (D) Air-sea heat flux; total (black) and shortwave (blue) components from NOC climatology (see supple-

mentary materials). NOC plus 40 W m^{-2} (NOC + 40) shows the range of uncertainty. Short, thick lines indicate average values from the R/V *Knorr* cruise. (E) Wind stress used to drive the model derived from WaveWatch III (see supplementary materials), eastward (black) and northward (blue) components. (F) Model stratification (average N^2 over layer) from runs with lateral density gradient (shaded gray) and without (purple) for 0- to 100-m (solid) and 100- to 300-m (dashed) layers. The shaded regions indicate a range of model solutions obtained by increasing the NOC heat flux by 40 W m^{-2} to account for uncertainty in the heat flux estimate. Red and green curves from observations in (B) are overlaid. (G) Model chlorophyll concentration in the upper 100 m (average), colored as in (F). Chlorophyll from the combination of observations in (C) is overlaid.

the absence of winds, ML eddies would overcome cooling-induced mixing and restratify the ML (that is, $S < 1$) when the cooling weakens beyond the -100 W/m^2 threshold. Down-front winds also counter restratification by ML eddies, and a similar scaling estimate (22) for the ratio of buoyancy fluxes due to down-front winds and ML eddies is given by $S_w = \frac{\tau}{0.06\rho_0 b_y H^2}$, where τ is the down-front surface wind stress. $S_w = 1$ corresponds to $\tau = 0.17 \text{ Pa}$.

Variations of parameters S and S_w can qualitatively explain the timing of the observed stratification during NAB08. Before yearday 70, strong surface cooling ($Q \sim -200 \text{ W/m}^2$) and down-front winds ($\tau = 0.2 \text{ Pa}$) easily overcame ($S > 1$, $S_w > 1$) restratification by ML eddies. Consequently, the ML remained well mixed. Near yearday 80, persistent eastward (down-front, $S_w > 1$) winds shifted westward (up-front, $S_w \sim 1$). Near yearday 100, surface cooling declined to below -100 W/m^2 ($S \leq 1$), which is roughly when ML stratification started to increase. Thus, theory suggests that the mixed layer restratified because of the decrease in surface cooling, aided by the switch in wind direction from eastward to westward. However, uncertainties in the scaling parameters in these simple formulas, as well as the complexities of their combined effects, require a more detailed evaluation of this hypothesis through modeling.

Modeling. We conducted a more quantitative analysis of this mechanism with the use of a 3D process-study ocean model (23) coupled to a single-species, light-limited phytoplankton model (supplementary materials sections 1.4 to 1.9). The domain, encompassing several of the many small fronts seen in Fig. 2B, is 480 km (north-south) by 96 km (east-west, periodic). The model is initialized in midwinter (yearday 30), with three fronts spanning the domain, and evolves for 120 days. By yearday 95, the start of our observations, the model has evolved to an equilibrium condition with little resemblance to the initial conditions (supplementary materials section 1.7 and fig. S6). The north-south average gradient b_y , wave number spectrum of b_y , and probability density function of b_y are close to those found along the observed transect in Fig. 2B (figs. S7 and S8). The model is slightly smoother than the observations, underestimating the mean square b_y by $\sim 50\%$, which would slow the model's restratification rate by $\sim 20\%$ (supplementary materials section 1.7), but this error is less than other uncertainties in the model.

The model is forced (Fig. 3, D and E, details in the supplementary materials) by a uniform surface wind stress and a surface heat flux from the NOC 1.1a climatology (16), which cools slightly more strongly toward the north. Bagniewski *et al.* (24) optimized an ecosystem model to fit

observations of light, chlorophyll, particulate carbon, nitrate, and silicate made by (or near) the NAB08 Lagrangian float. These data were sufficient to strongly constrain the phytoplankton model parameters. Model components related to nutrient limitation and grazing were removed, leaving a single group of modeled phytoplankton limited only by light. This is appropriate for the early part of the bloom where nutrient limitation and grazing play a minor role in the full model. Before the onset of the bloom, the average phytoplankton in the ML is restored toward a seed population with 0.08 mg m^{-3} of chlorophyll, based on observations at the start of NAB08. Details of the model and comparisons with data can be found in the supplementary materials and also in (24).

Results from four model simulations, two of which generate ML eddies due to the presence of a horizontal density gradient ("Gradient" cases in Fig. 3), and two control runs, which do not ("No Gradient" cases in Fig. 3), characterize the impact of ML eddies. Two ML eddy simulations using the NOC heat flux (supplementary materials and methods and figs. S5 and S6) and NOC plus 40 W/m^2 (Fig. 3D) span the uncertainty in heat flux. The controls are identical to these, but initialized with no lateral density gradients and thus have no ML eddies. Additional simulations vary wind and heat flux (supplementary materials section 2, fig. S9, and table S1).

The two ML eddy simulations (Fig. 3F, shaded) reproduce the two phases of stratification seen in the data. The first phase stratifies both the shallow and deep layers starting near yearday 90; the second phase strongly stratifies the shallow layer starting at yearday 110 to 120. These results agree with the observations to within the uncertainties due to heat flux (shading) and inter-annual variability [differences between NAB08 (red) and climatology (green)]. The slight divergence between the rate of increase of stratification in the upper and lower layers before day 120 is also reproduced in the model. They also reproduce the concurrent increase in chlorophyll (Fig. 3G, shaded) around yearday 110. After roughly yearday 130, the modeled and observed chlorophyll diverge due to the onset of silicate limitation, which is not included in the model. The "No Gradient" control cases, (Fig. 3F, purple) predict no increase in stratification until the heat flux turns positive near yearday 120 and no increase in chlorophyll (Fig. 3G, purple) until about yearday 130. These model simulations indicate that the initial stratification of the winter-time ML and the onset of the bloom before atmospheric warming are due to ML eddies arising from horizontal ML density gradients. In their absence, the stratification and bloom would be delayed by 20 to 30 days.

The North Atlantic spring bloom is patchy (25), as seen in a satellite chlorophyll image (Fig. 4B) and in the spread of the float and Seaglider chlorophyll data (Fig. 3C, compare orange dots

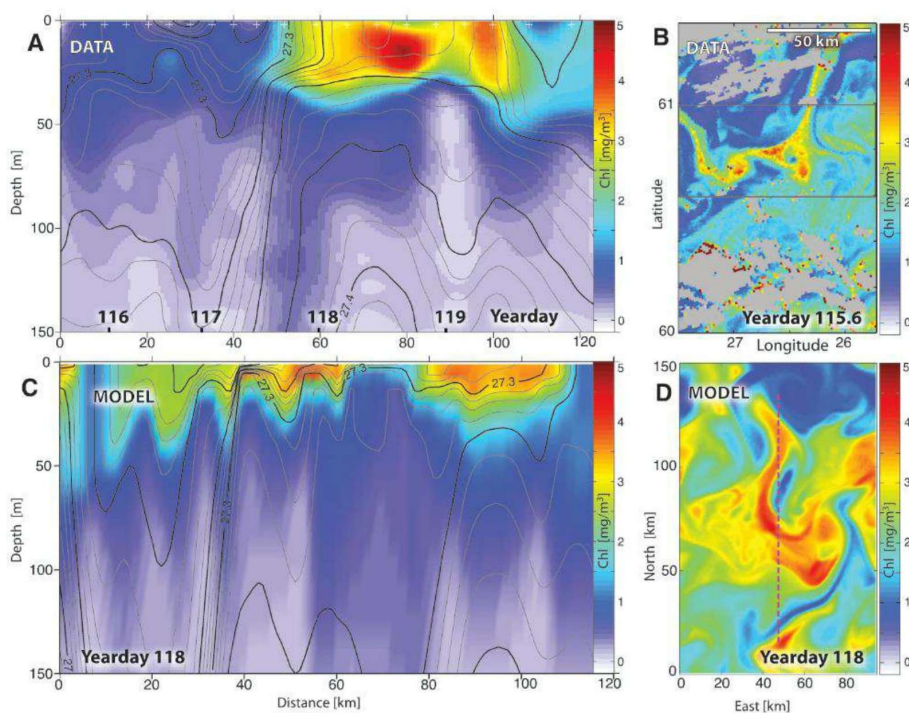


Fig. 4. Spatial structure of bloom in data (top row) and from model (bottom row). (A) Seaglider section, with chlorophyll (color) and potential density (contours). The finest contour interval is $0.00625 \text{ kg m}^{-3}$. Small white crosses mark profile locations. Data are smoothed over $\sim 7 \text{ km}$ horizontally. The horizontal axis is either distance along track (x axis) or time (black numbers). (B) MODIS satellite chlorophyll (yearday 115.6) multiplied by 1.92, the product of 1.5 (as in Fig. 3C) and 1.28, to account for the average chlorophyll increase between yeardays 115.6 and 118. Gray denotes clouds. (C) Model section across chlorophyll patches on yearday 118 plotted as in (A). (D) Model surface chlorophyll on yearday 118 plotted as in (B). The dashed vertical line shows the location of the section in (C).

and line). A measured vertical-horizontal section during bloom growth (Fig. 4A) shows steeply sloping density surfaces with regions of high and low stratification. Near-surface chlorophyll is greatest above the strong stratification (26), where the reduced depth of mixing leads to greater light exposure and more phytoplankton growth. A significant correlation exists between the spatial variations in chlorophyll and stratification in the upper 100 m (fig. S16) during the period of rapid growth (yeardays 110 to 116). A model section (Fig. 4C) shows similar vertical and horizontal structures. Plots of surface chlorophyll from data (Fig. 4B) and the model (Fig. 4D) show kilometer-scale filaments of high and low chlorophyll. Wave number spectra of chlorophyll (fig. S15) from the model and from the image in Fig. 4 have similar shapes: white at 100-km wavelength steepening to a -2 slope at the 10-km wavelength. Observations and simulations exhibit similar patchiness, with variations in stratification and phytoplankton biomass occurring on similar scales.

Discussion. Several recent studies have proposed alternate hypotheses for the initiation of spring blooms, but these theories do not explain our observations. Taylor and Ferrari (11) suggest that the bloom occurs when the ML turbulence decreases, allowing phytoplankton to grow within a deep, but weakly mixed layer. The vertical motion of the Lagrangian float directly measures the vertical velocity (27) and, thus, the intensity of the turbulence. The root mean square vertical velocity is strongly correlated with wind stress, neither of which decreased very much at the time of the bloom (fig. S17A). Furthermore, this mechanism acts by creating strong vertical gradients of plankton within the ML, which are not observed (supplementary materials section 5 and fig. S17B). Taylor and Ferrari (3) also suggest that symmetric instability (SI) could produce vertical stratification from horizontal gradients. However, the amount of stratification that SI could produce is very small compared with ML eddies (supplementary materials section 5.2). Furthermore, it is not dominant during the stratification phase, because the direction of the wind relative to front (28), surface cooling, and density-velocity structure are not favorable. Alternatively, Behrenfeld (29) suggests that a deepening ML dilutes plankton, decreasing the interaction rate between phytoplankton and zooplankton, reducing grazing, and thus allowing phytoplankton to grow. The NAB08 bloom occurs during a period of shallowing ML depth, so this mechanism does not apply.

Blooms driven by ML eddy restratification evolve differently from those driven by surface heating. The timing of a bloom caused by eddy restratification depends on the lateral density gradient, ML depth, the decrease in surface cooling below a threshold value, and wind speed and direction. In contrast, the timing of a bloom caused by surface heating depends on the onset of heating and on wind strength (mixing rate), but not

on wind direction. Differences in forcing functions between these two mechanisms suggest that the response of bloom onset to interannual and climatic changes could depend strongly on which mechanism prevails.

Blooms initiated by ML eddies can be expected to have patchier growth (fig. S12) and greater spatial heterogeneity at the 1- to 10-km scale than those initiated by surface heating, as the former introduces more horizontal gradients. This heterogeneity may allow a more diverse planktonic community with different species dominating in different patches (30). Even in midwinter, the ML eddies could generate intermittent patches of shallow stratification, as seen in the Argo float data (Fig. 3B), leading to enhanced winter productivity (29) and the maintenance of seed populations for the spring bloom (31).

The ML eddies that initiate the bloom also transport water vertically along the sloping isopycnals, bringing nutrient-rich water from across the entire wintertime ML intermittently into the euphotic zone (Fig. 4A). In contrast, blooms caused by surface heating alone will be confined to the shallow surface layer, limiting access to and shading the nutrient-rich waters below. Thus although our simulations do not include nutrient effects, we anticipate that enhanced nutrient fluxes into surface waters due to ML eddies will lead to an overall increase in carbon fixation.

Eddy restratification is effective in this area of the Icelandic basin due to the existence of deep (200 to 400 m) MLs and substantial (1 to 5×10^{-7} kg-m $^{-4}$) average lateral density gradients (Fig. 2, C and D). Analysis of Argo float data for the January-to-April period (Fig. 2, C and D) shows similar conditions across most of the subpolar North Atlantic, with the westward shallowing of MLs compensated by increasing lateral gradients. These conditions are widespread in the subpolar oceans, suggesting that the ML eddy processes described here play a major role in controlling bloom timing in the subpolar North Atlantic (2) and in similar blooms globally.

Roughly half (32) of global net photosynthesis occurs in the ocean, with upper-ocean stratification playing a key role in regulating productivity. The current generation of climate models control springtime ML stratification exclusively by vertical processes. These results suggest that lateral processes driven by horizontal density gradients also play an important role, as the results affirm the need for parameterizing these processes (33) and their importance in controlling ocean productivity and the timing of phytoplankton blooms (3, 9, 11, 34).

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Supplementary Materials

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Materials and Methods

Supplementary Text

Figs. S1 to S17

Table S1

References

Movie S1

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Structural Basis of Wnt Recognition by Frizzled

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Wnts are lipid-modified morphogens that play critical roles in development principally through engagement of Frizzled receptors. The 3.25 angstrom structure of *Xenopus* Wnt8 (XWnt8) in complex with mouse Frizzled-8 (Fz8) cysteine-rich domain (CRD) reveals an unusual two-domain Wnt structure, not obviously related to known protein folds, resembling a “hand” with “thumb” and “index” fingers extended to grasp the Fz8-CRD at two distinct binding sites. One site is dominated by a palmitoleic acid lipid group projecting from serine 187 at the tip of Wnt’s thumb into a deep groove in the Fz8-CRD. In the second binding site, the conserved tip of Wnt’s “index finger” forms hydrophobic amino acid contacts with a depression on the opposite side of the Fz8-CRD. The conservation of amino acids in both interfaces appears to facilitate ligand-receptor cross-reactivity, which has important implications for understanding Wnt’s functional pleiotropy and for developing Wnt-based drugs for cancer and regenerative medicine.

Wnts (Wingless and Int-1) are central mediators of vertebrate and invertebrate development, owing to their influences on cell proliferation, differentiation, and migration (1–5). Wnts, which are ~350-residue secreted, cysteine-rich glycoproteins that activate cell surface receptors on responder cells to initiate at least three different signaling pathways, including the “canonical” β -catenin pathway and the “noncanonical” planar cell polarity and Ca^{2+} pathways (1, 4–6). The seven-pass transmembrane receptor Frizzled (Fz) is critical for nearly all Wnt signaling, and the N-terminal Fz cysteine-rich domain (CRD) serves as the Wnt binding domain. In addition to Fz, the Wnt/ β -catenin pathway requires the low-density lipoprotein receptor–related proteins 5 and 6 (Lrp5/6) co-receptors (7). Wnt signaling is also regulated by several alternative receptors, such as Ryk and Ror2, and by secreted antagonists (8) that directly interact with Wnts, such as Wnt-inhibitory factor (WIF-1) (9), or engage Wnt receptors, such as Dickkopf (Dkk) (10) and Kremen (Krm) (11, 12). Dysregulation of the Wnt/Fz system is associated with a variety of human hereditary diseases, and modulation of Wnt signaling is actively targeted for cancer, regenerative medicine, stem cell therapy, bone growth, and wound healing (13–17).

There exists no structural information for Wnts: Their primary sequences are not clearly related to any known protein folds. Wnts are hydrophobic owing to the posttranslational addition of palmitate and/or palmitoleic acid to one or two residues (Cys⁷⁷ and/or Ser²⁰⁹ in Wnt3a) (18, 19). Acylation is necessary for both Wnt intracellular trafficking and its full activity when secreted, but its precise role in Wnt action remains unclear. It has been speculated that the Wnt lipid group(s) may directly engage the Fz-CRD (20) and could

also mediate binding to WIF (21, 22). Genetic evidence suggests that Wnt-secreting cells require the action of the acyltransferase Porcupine for Wnt palmitoylation (23). As Wnts are morphogens, the acylation is thought to localize Wnts to cell membranes, and circulating Wnts may be bound to carrier proteins that shield the lipid from solvent (24). Wnt palmitoylation has complicated expression and purification of recombinant material (25). As a result of these technical difficulties, relatively few detailed structure-function studies of Wnts have been carried out that shed light on how Wnts engage Fz.

Current structural knowledge of Frizzled receptors is limited to the unliganded Fz8-CRD and the secreted CRD antagonist sFRP (secreted Frizzled-related proteins), which are ~120-residue, primarily α -helical proteins (26). Mutational mapping studies of *Xenopus* Wnt8 (XWnt8) interactions with Fz8, Fz4, and *Drosophila* Fz2 (DFz2) identified several potential patches on the CRD

important for binding (26–28). Potential Wnt-binding patches on the Fz4-CRD also appear to mediate binding to the Norrie disease protein Norrin, which is a cysteine-knot growth factor unrelated in sequence to Wnt, that has been shown to activate Fz4 (28). With respect to Fz activation, the molecular mechanisms are unknown. Although Fz proteins share several features of G protein-coupled receptors (GPCRs), they lack hallmark characteristics of prototypical GPCRs (4, 29). Nevertheless, some principles of transmembrane signaling by GPCR may be relevant (30).

A confounding feature of the Wnt/Fz system has been how functional specification is achieved when each Wnt can engage multiple Fz receptors, and each Fz can respond to multiple Wnts (5, 27, 28, 31–34). This pleiotropy confounds interpretation of in vivo functional experiments. Fz receptors and Wnt ligands have not been unambiguously matched, and it is unclear if mono-specific Wnt/Fz pairs are responsible for certain biological effects and diseases (31, 32). Structural information on Wnt and Wnt/Fz interactions can shed light on the critical issues of Wnt/Fz specificity and the functional role of Wnt acylation and can begin to give insight into a mechanism of receptor activation. Here, we present the structure of XWnt8 in complex with the Fz8-CRD to a resolution of 3.25 Å.

The XWnt8 complex with Fz8-CRD. We screened a variety of vertebrate and invertebrate Wnts for expression and found that XWnt8 was expressed at high levels and could be purified as a complex with several Fz-CRD proteins. XWnt8 is advantageous for structural studies, as it has served as a model system to study Wnt/Fz interactions because it binds to and activates mammalian Fz (27). A key enabling finding was that coexpression of XWnt8 with an Fz8-CRD-Fc fusion allowed efficient affinity-based purification

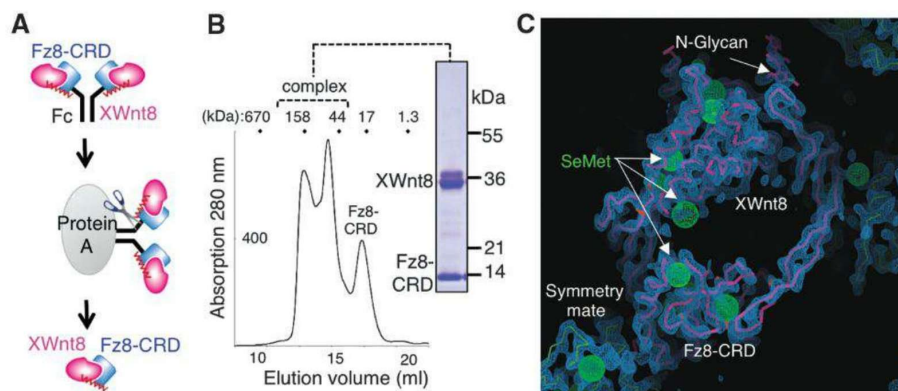


Fig. 1. Formation of the XWnt8 complex with mouse Fz8-CRD for structure determination. **(A)** Strategy for purification of the XWnt8/Fz8-CRD complex. The mouse Fz8-CRD was coexpressed as an Fc-fusion protein with XWnt8 in *Drosophila* S2 cells, and the complex was captured with protein-A. The XWnt8/Fz8-CRD complex was eluted from the resin with 3C protease, which cleaved the Fz8-CRD from the Fc. **(B)** The complex was then purified by gel filtration chromatography. The doublet band for XWnt8 represents glycosylation heterogeneity. **(C)** Initial density-modified electron density map calculated with experimental phases derived from selenomethionine sites (green spheres). N-Glycan evident in the experimentally phased map is labeled. The initial backbone trace built into this map is shown within the electron density, along with neighboring symmetry mates. See also table S1 and fig. S1 for electron density of the refined structure.

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of XWnt8/Fz8-CRD complexes in the absence of detergent. In contrast, purification of Wnt alone requires detergent, suggesting that binding to the Fz8-CRD shields the Wnt lipid from aqueous solvent (Fig. 1, A and B). The XWnt8/Fz8-CRD complex eluted from gel filtration as interconverting oligomeric forms with molecular masses ranging from ~50 to ~200 kD (Fig. 1B).

We crystallized the glycosylated XWnt8/Fz8-CRD complex in detergent-free buffers and obtained a native x-ray data set to a resolution of 3.25 Å (table S1). Experimental phases were determined by means of isomorphous replacement and anomalous scattering difference methodologies with crystals derived from material expressed in S2 cells supplemented with selenomethionine (table S1). The experimental phases yielded an excellent electron density map in which XWnt8 could be traced, the Fz8-CRD located (Fig. 1C), and the complex structure refined (fig. S1). The amino acid register of XWnt8 was confirmed with the selenium sites as guides (Fig. 1C), as well as the locations of disulfide bridges, N-linked glycans (fig. S1), and the lipid group.

The complex structure is a distinctive donut shape (Fig. 2, A and B) in which XWnt8 appears to grasp the Fz8-CRD at two opposing sites using extended thumb and index fingers projecting from a central “palm” domain, to contact “site 1” and “site 2,” respectively, burying a total of ~2000 Å² of surface area. Neither the structure of XWnt8 nor the manner of Fz binding clearly resembles that of known protein folds or complexes, respectively. XWnt8 comprises an N-terminal α -helical domain (NTD) from residues ~32 to 250 (helices A through G) that contains the lipid-modified thumb, and a C-terminal cysteine-rich domain (CTD) from residues 261 to 338. Each domain forms a distinct interaction with the Fz8-CRD, whose conformation is essentially unchanged compared to the unliganded structure (fig. S2A), leaving a large hole in the center of the complex (Fig. 2, A and B, and fig. S2B). The XWnt8 NTD is composed of a seven- α -helical bundle palm, containing two large interhelical loop insertions that are stabilized by four disulfide bonds (Fig. 2, A and D). The principal feature of the CTD is a long 38-amino acid β -strand hairpin that is also stabilized by an extensive network of disulfide bonds. The distinct structural subdomains associate through a small interaction patch between the AB loop of the NTD and a small helix (helix F) in the CTD. There is clear electron density for high-mannose glycan additions at two of the three asparagine-linked glycosylation sites on XWnt8, Asn¹⁰⁴, and Asn²⁶³ (Fig. 2C and fig. S1B).

The XWnt8 lipid directly engages a groove on the Fz8-CRD. The functional role for lipid modification of Wnts is unknown, but has been shown to be necessary for full biological activity (18). The structure shows XWnt8 lipidation directly involved in Fz8-CRD binding in binding site 1 (Figs. 2A and 3). A 15 Å-long tube of continuous electron density is connected to the hydroxyl group of Ser¹⁸⁷ (Fig. 3A), which is located

at the tip of the thumb projecting from the XWnt8 NTD. The length of the electron density corresponds to that of a 14-carbon lipid chain. The lipid dominates the contact interface, burying ~580 Å² of total surface area (330 Å² from the lipid, 250 Å² from the CRD), contacting 9 Fz8 residues, and completely traversing the cleft on the Fz8-CRD surface (Fig. 3B). The lipid electron density is consistent with a 16-carbon palmitoleic acid (or derivative thereof) modification to XWnt8 where the terminal two carbons of the acyl chain have exited the CRD groove and do not show ordered electron density. Wnt has been reported to be acylated with either a saturated palmitic acid or a monounsaturated palmitoleic acid (23). We could not unambiguously determine the chemical identity of the lipid on XWnt8 using mass spectrometry (Fig. 3A). However, based on identification of the lipid attached to the corresponding Ser²⁰⁹ of mouse Wnt3a as palmitoleic acid, we assigned the lipid attached to XWnt8 Ser¹⁸⁷ as

palmitoleic acid—but it is formally possible that the lipid is palmitic acid. Serine acylation in the complex structure also resolves uncertainty regarding the location of the lipid attachment sites on Wnts. Both conserved Ser²⁰⁹ and Cys⁷⁷ residues have been reported as acylation sites on mouse Wnt3a, and it has been speculated that other Wnts are acylated at one or both of the corresponding positions (18, 19, 23). In XWnt8, we find that Cys⁵⁵, the Cys residue analogous to Cys⁷⁷ in Wnt3a, is engaged in a disulfide bond that will be conserved across all Wnts (Fig. 2D and fig. S3) and so cannot serve as a lipid addition site. Therefore, the conserved Ser (corresponding to Ser¹⁸⁷ in XWnt8) appears to be the consensus acylation site.

The cleft on the Fz8-CRD surface that is traversed by the lipid is made up of helix B, helix D, and the DE loop (Fig. 3B and table S2) and is lined with hydrophobic amino acids that form extensive van der Waals interactions with the lipid (Fig. 3B and table S2). The high degree of

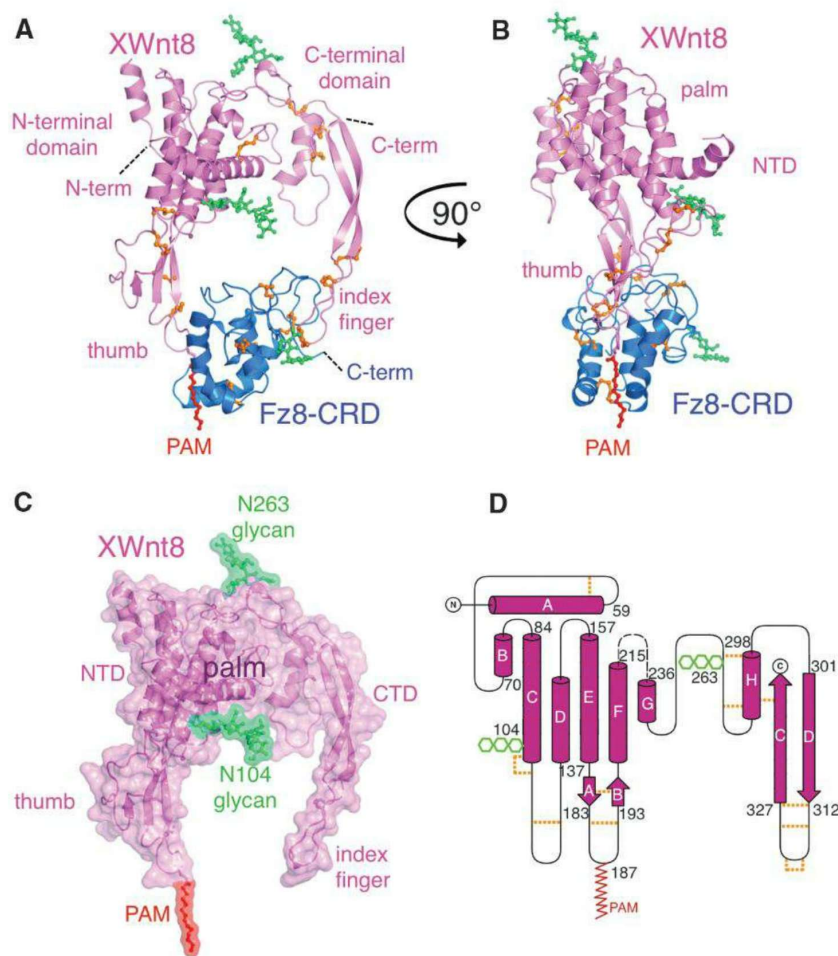


Fig. 2. Overall structure of XWnt8 in complex with Fz8-CRD. Ribbon models of XWnt8 (violet) and Fz8-CRD (blue) as viewed “face on” (A) and “side on” (B). N-Linked glycans are drawn as green sticks; disulfide bonds are drawn as orange sticks. (C) Surface representation of XWnt8 after removal of the Fz8-CRD from the complex structure. The extended palmitoleic acid (PAM) group is shown in red extending from the Wnt thumb. See also fig. S5 for mapping of the potential Lrp5/6 binding site. (D) Secondary-structure diagram of the XWnt8 fold. Disulfide connectivity is indicated by orange lines, visible N-glycan addition sites by green shapes, PAM addition site by the red zigzag line. See also fig. S2 for images of the bound versus unbound structure of the Fz8-CRD, and a molecular surface of the entire complex.

conservation of apolar amino acids in the region of the CRD contacting the acyl group implies that the lipid-binding site is conserved in other Fz-CRD proteins (Fig. 3C and fig. S4). The conservative substitutions seen for these residues in other Fz-CRD proteins could modulate lipid-binding affinity and impart a degree of Wnt specificity (Fig. 3C). The driving force for lipid binding appears to be the hydrophobic effect combined with shape complementarity of the lipid-CRD interface, where the lipid and apolar Fz-CRD core residues are driven to associate by solvent exclusion. Although $\sim 60\%$ of the total accessible surface area ($\sim 530 \text{ \AA}^2$) of the lipid is buried when bound to the Fz8-CRD, one face and the distal two or three carbon atoms of the lipid are still exposed to solvent. These exposed regions, $\sim 200 \text{ \AA}^2$ of hydrophobic surface, would be highly energetically unfavorable in aqueous solvent and may still require shielding.

Fz-CRD interactions with the Wnt lipid are highly conserved. Although the site 1 interaction appears to be mediated largely by the lipid on Wnt, thumb loop amino acids (residues 182 to 188) form protein-protein contacts with the Fz8-CRD that account for an additional $\sim 600 \text{ \AA}^2$ of buried surface area (Fig. 3C). At the extreme tip of the thumb loop (residues 186 to 188), several main-chain van der Waals contacts are formed with the Fz8-CRD that would have limited capacity to contribute to ligand specificity (Fig. 3C). At the base of the thumb loop, XWnt8 Lys¹⁸² forms a salt bridge with the Fz8 Glu⁶⁴ and a hydrogen

bond with Fz8 Asn⁵⁸. Lys or Arg is conserved at this corresponding position in all Wnts, and Glu or Asp is conserved at the Glu⁶⁴ position in 8 of 10 mammalian Fz-CRD proteins (table S2 and figs. S3 and S4). However, the substitution of Thr and Ile in Fz3 and Fz6, respectively, raises the possibility of some degree of ligand specificity modulated through this interaction. We surmise that the principal driving force for the site 1 binding is the lipid-in-groove contact, with the residues at the base of the thumb contributing secondarily.

The highly exposed structural disposition of the lipid attachment site has several important implications. First, it suggests that lipid attachment may not be integral to the tertiary structural stability of the folded Wnt molecule. Clearly, acylation is necessary for proper secretion of Wnts, and our complex structure also reveals its centrality in Fz binding. But it should be possible to create viable Wnt protein therapeutics by genetically engineering “lipid-free” water-soluble Wnts through affinity maturation of Fz-contacting residues at the tip of the Wnt thumb. Second, the highly exposed position of the lipid suggests it would require sequestration from aqueous solvent during expression and transport, such as with carrier proteins (24). In Wnt’s role as a morphogen, it has been suggested that Wnts may use acylation to partition into the cell membrane to increase local concentrations and restrict availability to specific target tissue (18, 19). The XWnt8 structure supports this idea in that the lipid is ac-

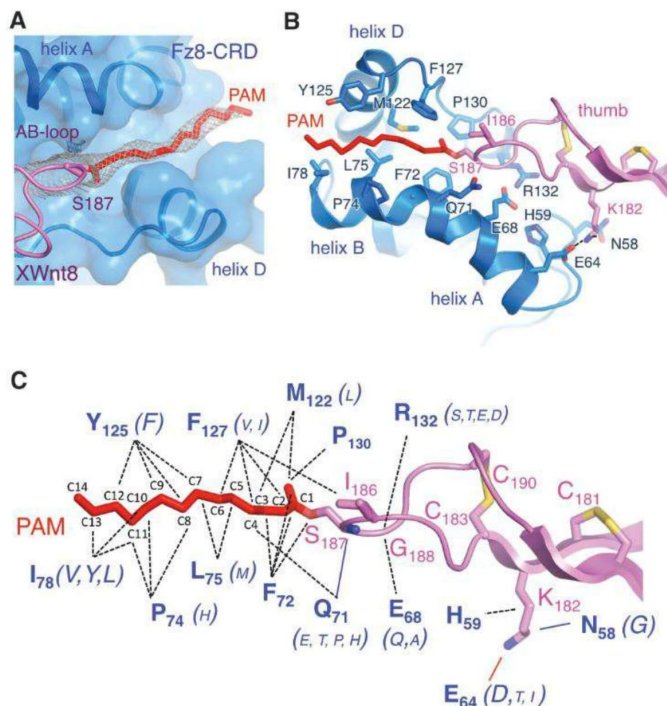
cessible (Fig. 2C), ideally positioned for anchoring Wnt to the plasma membrane.

A second XWnt8/Fz8-CRD binding interface.

The site 2 interaction is on the opposite side of the Fz8-CRD from site 1 (Fig. 2A) and is composed of residues between the Cys³¹⁵-Cys³²⁵ disulfide at the tip of the XWnt8 CTD index finger, engaging in hydrophobic contacts within a depression between interhelical loops on the CRD (Fig. 4, A and B, and table S2). The site 2 interface buries a total of $\sim 800 \text{ \AA}^2$ ($\sim 375 \text{ \AA}^2$ CRD, $\sim 423 \text{ \AA}^2$ XWnt8) and despite the “knob-in-hole” binding mode (Fig. 4A), exhibits poor overall shape complementarity ($Sc = 0.48$). The XWnt8 index finger presenting the site 2 residues is a long, twisted β strand, rigidified by a ladder of disulfide bonds, and spans from Arg³⁰¹ to the C-terminal Cys³³⁸ (Fig. 2D). In site 2, the finger loop positions hydrophobic residues Cys³¹⁵, Phe³¹⁷, Trp³¹⁹, an unusual tandem Cys³²⁰-Cys³²¹ disulfide bond, and Val³²³ to form the major van der Waals interactions with main-chain and apolar residues on the Fz8-CRD (Fig. 4, B and C). The XWnt8 Trp³¹⁹ side chain at the tip of the finger loop occupies a pocket on the Fz8-CRD surface and engages primarily the main chain of Fz8-CRD residues 150 to 152. The XWnt8 site 2 contact residues are highly conserved or invariant in all Wnts (fig. S3). In the Fz8-CRD Tyr⁴⁸ and Cys¹⁴⁸, conserved residues form van der Waals interactions with XWnt8 (fig. S4). As for site 1, Wnt and Fz contact residues are conserved apolar amino acids (Fig. 4C and fig. S3). Notably, several Fz8-CRD contacts are substituted in other Fz-CRDs and thus could contribute to Wnt subtype preferences. For example, Met¹⁴⁹ at the center of site 2 is conserved in 5 of 10 mammalian Fz-CRD proteins, but is substituted by Val, Glu, or Asp in Fz1, 2, 3, 6, and 7.

“Mini-XWnt8” autonomously engages the Fz8-CRD in a receptor-specific manner. Given the technical difficulties of expressing recombinant Wnts, there is a dearth of structure-function data, or biochemical measurements between Wnt and Fz. Here, guided by the structure of the complex, we engineered a biochemically tractable version of XWnt8 to determine three previously unknown interaction parameters: (i) the degree to which XWnt8 site 1 versus site 2 binding determines Fz specificity; (ii) if the site 1 and 2 interactions can occur independently, or whether both sites are reliant on simultaneous engagement to achieve productive binding; and (iii) measurement of an accurate binding affinity of site 2 alone. For the first experiment, we displayed a water-soluble, C-terminal 90-amino acid subdomain of XWnt8, containing the site 2 binding index finger (which we term “mini-Wnt”) on yeast. We tested Fz1, 2, 4, 5, 7, and 8 CRD for binding and clearly observed that mini-XWnt8 was stained by fluorescence-activated cell sorting (FACS) with several Fz-CRD (Fz4, Fz5 and Fz8) that were presented as fluorescent tetramers by forming complexes with streptavidin-phycoerythrin (PE) (Fig. 5A). Notably, we observed stronger

Fig. 3. Acylation of the XWnt8 thumb loop mediates site 1 binding to Fz8-CRD. (A) Shape and chemical complementarity of the lipid-CRD interaction is evident when the electron density of the lipid modification (red lipid in gray mesh, σ_A -weighted $2F_o - F_c$ map contoured at 0.8σ) at Ser¹⁸⁷ of XWnt8 (violet) is shown together with the molecular surface of the site 1 groove in the Fz8-CRD (blue). (B) Amino acid interactions mediating recognition of the XWnt8 thumb by the Fz8-CRD at site 1 (table S2). Several hydrogen bonds are drawn as dashed lines. (C) Site 1 recognition occurs largely through chemically or strictly conserved Wnt and Fz amino acids (see also figs. S3 and S4). Residues of the Fz8-CRD that contact XWnt8 or the lipid are indicated with blue labels. Alternative residues at each position in other Fz-CRD proteins are indicated within parentheses. The relative font sizes of the different amino acids within the parentheses reflects an approximate percentage of the 10 Fz receptors that use that amino acid at the respective position. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.



Implications of the XWnt8 complex with Fz8-CRD for functional pleiotropy. The potential combinatorial complexity of 19 mammalian Wnts engaging 10 Fz receptors raises an important question: Do cross-reactive or specific Wnt/Fz pairs contribute to distinct biological, and disease-related functions? From the XWnt8/Fz8-CRD complex structure, it is apparent that most Wnt/Fz-CRD contacts in both site 1 and site 2, including the lipid interactions, involve either strictly or chemically conserved residues. Thus, we conclude that the site1/site2 Wnt/Fz interaction chemistry is incompatible with monospecificity and therefore a binary or highly restricted ligand-receptor matching code most likely does not exist. Clearly, however, some studies, including those shown here with mini-Wnt (Fig. 5), have shown that Wnts and Fz are not broadly degenerate (27, 32, 33). Rather, it appears that most Wnts can engage

possessing different binding affinities. With the structure of a Wnt/Fz-CRD complex, it is feasible to attempt to engineer Wnts that are monospecific for Fz in order to probe the role of Wnt/Fz specificity in function.

A Fz8-CRD

B

C

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the thumb and finger loops, the core of the NTD helical bundle, and a large continuous patch at the “top” of XWnt8 (fig. S5). This patch is composed of ~10 residues derived from three discontinuous regions of sequence primarily on solvent-exposed interhelical loops: residues 216 to 219, 249 to 252, and 256 to 259. The location of the conserved patch at the opposing end of XWnt8 from the Fz-CRD binding region, and its solvent exposure, lead us to highlight this region as a potential Lrp5/6 and/or Ryk binding site on Wnts that would enable bridging with Fz to form a ternary complex.

Implications of the XWnt8/Fz-CRD ectodomain complex for Fz receptor activation. What does the structure of the XWnt8/Fz8-CRD complex indicate about Fz receptor activation? Wnt receptor clustering appears to be crucial for signaling and Dishevelled signalosome assembly (40), but given current uncertainty as to the compositions of Fz signaling complexes in canonical and noncanonical Wnt signaling, it is difficult to speculate on oligomerization-based signaling models (4, 35). Because Wnts activate Fz in the presence (canonical signaling, e.g., Wnt1, Wnt3a, Wnt8) or absence (noncanonical signaling, e.g., Wnt4, Wnt5a, Wnt11) of Lrp5/6 (4), cross-linking of Fz with Lrp5/6 does not appear to be absolutely required for all types of Fz signaling. Therefore, an activation mechanism may exist whereby Wnt/Fz engagement alone is sufficient for signaling in some settings, and this could involve dimerization (41). Of notable relevance is that Wnt5a activates Ror2, which is a tyrosine kinase (TK) receptor containing a CRD in its ex-

tracellular region that presumably serves as the Wnt-binding domain. Because TK receptors are activated by ligand-induced homo- or heterodimerization (e.g., epidermal growth factor receptor) (42), it is plausible that Wnt5a activates Ror2 through dimerization via the CRD. By extension, if the structural mode of Wnt/CRD interaction for Ror2 is analogous to that seen here for Wnt/Fz, Wnt may activate Fz partly through receptor dimerization. Canonical Wnts appear to have evolved an additional binding site to recruit Lrp5/6 into this signaling complex. Recent studies implicating Wnt5a as heterodimerizing Ror2 with Fz to initiate noncanonical signaling through a TK-independent mechanism further suggest the possibility of Wnt-induced heterodimerization of signaling receptors (35).

Although we see evidence of higher-order species of the XWnt8/Fz8-CRD complex in solution that would support an oligomerization model (Fig. 1B), we do not find evidence of a symmetric dimer in the crystal. We do, however, see a third site of contact in the crystal mediating an asymmetric Wnt/Fz dimer that we term “pseudo-site 3” (fig. S6). The interface is formed by one Wnt molecule binding to the composite Wnt-lipid/CRD site 1 surface presented by a different Wnt/Fz binary complex (fig. S6, A and B). The physiological relevance of pseudo-site 3 is not known, but it is the largest interaction surface in the crystal and buries ~200 Å² of lipid surface left exposed to solvent in site 1 (fig. S6B). Although the residues on the Fz8-CRD contacting XWnt8 in pseudo-site 3 are mostly conserved

(fig. S4), the residues on XWnt8 in the pseudo-site 3 interface are less conserved among Wnts (fig. S3). The asymmetric nature of pseudo-site 3 in the crystal lattice generates repeating units of self-associating binary complexes (fig. S6C). Because Dishevelled signalosome assembly is itself based on DIX-dependent “head-to-tail” polymerization (41, 43), it is intriguing to speculate whether pseudo-site 3 provides an underlying basis for both phenomena—ligand-induced receptor clustering and signalosome assembly. The structure of the XWnt8/Fz8-CRD pair presented here serves as a conceptual and technical framework to address remaining questions about the nature of canonical versus noncanonical signaling complexes, and how Wnt recognition by Fz is coupled to receptor activation.

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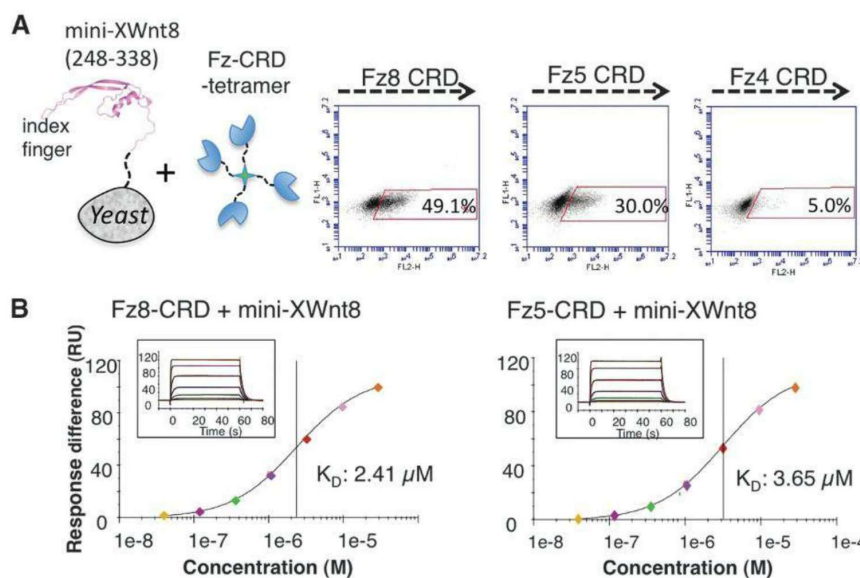


Fig. 5. “Mini-Wnt” discriminates between different Fz-CRD proteins and engages site 2 independently of site 1. **(A)** The C-terminal 90 amino acids of XWnt8 were displayed on the surface of yeast and shown to bind robustly to Fz5- and Fz8-CRD, and weakly to F4-CRD, by FACS analysis using fluorescent Fz-CRD–streptavidin-PE tetramers. The cartoon of mini-Wnt displayed on yeast depicts the C-terminal 90 amino acids seen in the structure. The population of mini-Wnt yeast stained by the Fz-CRD tetramers by FACS was ~50, ~30, and ~4% for Fz8, Fz5, and Fz4-CRD, respectively. **(B)** Surface plasmon resonance analysis of mini-XWnt8 binding to Fz5- and Fz8-CRD immobilized on a Biacore T100 sensor chip. Dose-response titrations are plotted of the equilibrium binding experiments; the insets of each panel show the titration data and that each concentration reached steady state.

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the discovery and use of mini-Wnt. K.C.G. is a cofounder of Eleven Biotherapeutics, which is engaged in the rational design of protein therapeutics.

Supplementary Materials

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Materials and Methods

Figs. S1 to S6
Tables S1 and S2
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Evolution and Functional Impact of Rare Coding Variation from Deep Sequencing of Human Exomes

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As a first step toward understanding how rare variants contribute to risk for complex diseases, we sequenced 15,585 human protein-coding genes to an average median depth of 111× in 2440 individuals of European ($n = 1351$) and African ($n = 1088$) ancestry. We identified over 500,000 single-nucleotide variants (SNVs), the majority of which were rare (86% with a minor allele frequency less than 0.5%), previously unknown (82%), and population-specific (82%). On average, 2.3% of the 13,595 SNVs each person carried were predicted to affect protein function of ~313 genes per genome, and ~95.7% of SNVs predicted to be functionally important were rare. This excess of rare functional variants is due to the combined effects of explosive, recent accelerated population growth and weak purifying selection. Furthermore, we show that large sample sizes will be required to associate rare variants with complex traits.

Understanding the spectrum of allelic variation in human genes and revealing the demographic and evolutionary forces that shape this variation within and among populations are major aims of human genetics research. Such information is critical for defining the architecture of common diseases, identifying functionally important variation, and ultimately facilitating the interpretation of personalized disease risk profiles (1–3). To date, surveys of human variation have been dominated by studies of single-nucleotide polymorphisms (SNPs) genotyped using high-density arrays composed of common

variants (4–6). Although these projects have substantially improved our knowledge of common allelic variation and enabled genome-wide association studies (GWAS), they have been generally uninformative about the population genetics characteristics of rare variants, defined here as a minor allele frequency (MAF) of less than 0.5%.

Rare genetic variants are predicted to vastly outnumber common variants in the human genome (7, 8). By capturing and sequencing all protein-coding exons (i.e., the exome, which comprises ~1 to 2% of the human genome), exome sequencing is a powerful approach for discovering rare variation and has facilitated the genetic dissection of unsolved Mendelian disorders and the study of human evolutionary history (9–14). Rare and low-frequency (MAF between 0.5 and 1%) variants have been hypothesized to explain a substantial fraction of the heritability of common, complex diseases (15). Because common variants explain only a modest fraction of the heritability of most traits (16, 17), the National Heart, Lung, and Blood Institute (NHLBI) recently sponsored the multicenter Exome Sequencing Project (ESP) to identify previously unknown genes and molecular mechanisms underlying complex heart, lung, and blood disorders by sequencing the exomes of a large number of individuals measured for phenotypic traits of substantial public health importance (e.g., early-onset myocardial infarction, stroke, and body mass index).

Data generation and variant discovery. A total of 63.4 terabases of DNA sequence was generated at two centers with three complementary definitions of the exome target and two different capture technologies (18). We sequenced samples from 15 different cohorts in the ESP to an average median depth of 111× (range of 23× to 474×). We found no evidence of cohort- and/or phenotype-specific effects, or other systematic biases, in the analysis of the filtered single-nucleotide variant (SNV) data (figs. S1 to S7). Exomes from related individuals were excluded from further analysis (fig. S8), resulting in a data set of 2440 exomes. We inferred genetic ancestry by using a clustering approach (18) and, unless otherwise noted, focused the remaining analyses on the inferred 1351 European-American (EA) and 1088 African-American (AA) individuals. We subjected the 563,698 variants in the intersection of all three capture targets to standard quality-control filters (18), resulting in a final data set of 503,481 SNVs identified in 15,585 genes and 22.38 Mb of targeted sequence per individual. We assessed data quality and error rates by several orthogonal methods (18). About 98% (941/961) of all variant sites that were experimentally tested were confirmed, including 98% (234/238) of singletons, 98% (678/693) of nonsingleton SNV sites with a MAF < 10%, and 97% (29/30) of SNV sites with a MAF ≥ 10%.

The vast majority of coding variation is rare and previously unknown. We observed a total of 503,481 SNVs and 117 fixed non-reference sites, of which 325,843 and 268,903 were found in AAs and EAs, respectively (fig. S9A). Excluding singletons, ~58% of SNVs were population-specific (93,278 and 32,552 variants were uniquely observed in AAs and EAs, respectively), and the vast majority of these variants were rare (fig. S9B). Most SNVs (292,125 or 58%) were nonsynonymous, including 285,960 missense variants and 6165 nonsense variants (fig. S9C). Synonymous variants accounted for 38% (188,975) of all SNVs (fig. S9C), with the remaining 4% of SNVs (22,381) located in either splice sites or targeted noncoding regions. The majority of SNVs (411,084; 82%) were previously unknown, with more novel SNVs observed in AAs (240,341) than in EAs (204,415), although the proportion of SNVs that were novel was higher in EAs compared with AAs (76.0% versus 73.8%; $\chi^2 = 398.3$, $df = 1$, $P < 10^{-16}$). About 98% (402,813) of novel SNVs were rare, and 48.9% of all novel, rare SNVs were nonsynonymous.

The AA and EA sample sizes provided ~90% power to detect variants with a MAF ≥ 0.1% and nearly 100% power to detect common variants

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(MAF $\geq 5\%$) (tables S1 and S2 and fig. S10). With our large sample size, the proportion of singletons identified rapidly decreased to a nearly constant rate of new singleton discovery such that each additional exome contributed ~ 200 novel SNVs (fig. S11). The number of SNVs per gene rapidly stabilized for common variants in small sample sizes (~ 100 individuals), whereas the number of rare variants continued to increase linearly (fig. S12).

Of the total SNVs, 57% (285,857) were singletons, and SNVs with three or fewer minor alleles accounted for 72% of all variants (fig. S9D). The proportion of singletons observed in AAs (49.3%, $n = 140,818$) was lower than that observed in EAs (50.7%, $n = 144,821$), but the overall site frequency spectra (SFS) and the SFS for both AAs and EAs are highly skewed, exhibiting a large excess of rare variants relative to the standard neutral model (19) (fig. S9D). The skew of the SFS was greater for EAs than AAs, and, at equal sample sizes, the odds that a SNV

was a singleton were 1.7 times greater for EAs than AAs. Consistent with these observations, Tajima's D was highly negative for both EAs (-2.12) and AAs (-2.10) and dropped precipitously as sample size increased (fig. S9E), highlighting that sequencing a large number of individuals provides unique information about recent demographic history (13, 20, 21).

To identify putatively functional variation, we applied four popular methods applicable to nonsynonymous variants (PolyPhen2, SIFT, a likelihood ratio test, and MutationTaster) and three conservation-based methods applicable to all types of variants [GERP, PhyloP, and a novel population genetics approach that combines conservation information with the SFS that we designate SFS-Del (18)]. About 47% of all SNVs (74% of nonsynonymous and 6% of synonymous variants) are predicted to be deleterious by one or more method (Fig. 1A); however, overlap among methods is modest. For example, only

1% of nonsynonymous variants are predicted to be functional by all seven methods, and variants predicted by any single approach are likely to have a high false-positive rate (Fig. 1A). Therefore, we used a conservative majority rule approach and deemed nonsynonymous variants predicted by at least four of the seven applicable methods and synonymous sites predicted by at least two of the three applicable methods (fig. S13) to be putatively functional. In total, 16.9% of SNVs (85,224) meet this criteria, of which 81,170 were nonsynonymous SNVs. About 95.7% (81,555) of all SNVs conservatively predicted to be functional were rare, and the odds ratio (OR) that rare variants are functional compared with variants with a MAF $> 0.5\%$ is 4.2 [95% confidence interval (CI) from 4.0 to 4.3; Fisher's exact test; $P < 10^{-15}$], underscoring the potential impact of rare variants on health-related traits.

Patterns of coding variation by gene and pathway. The median number of SNVs per gene

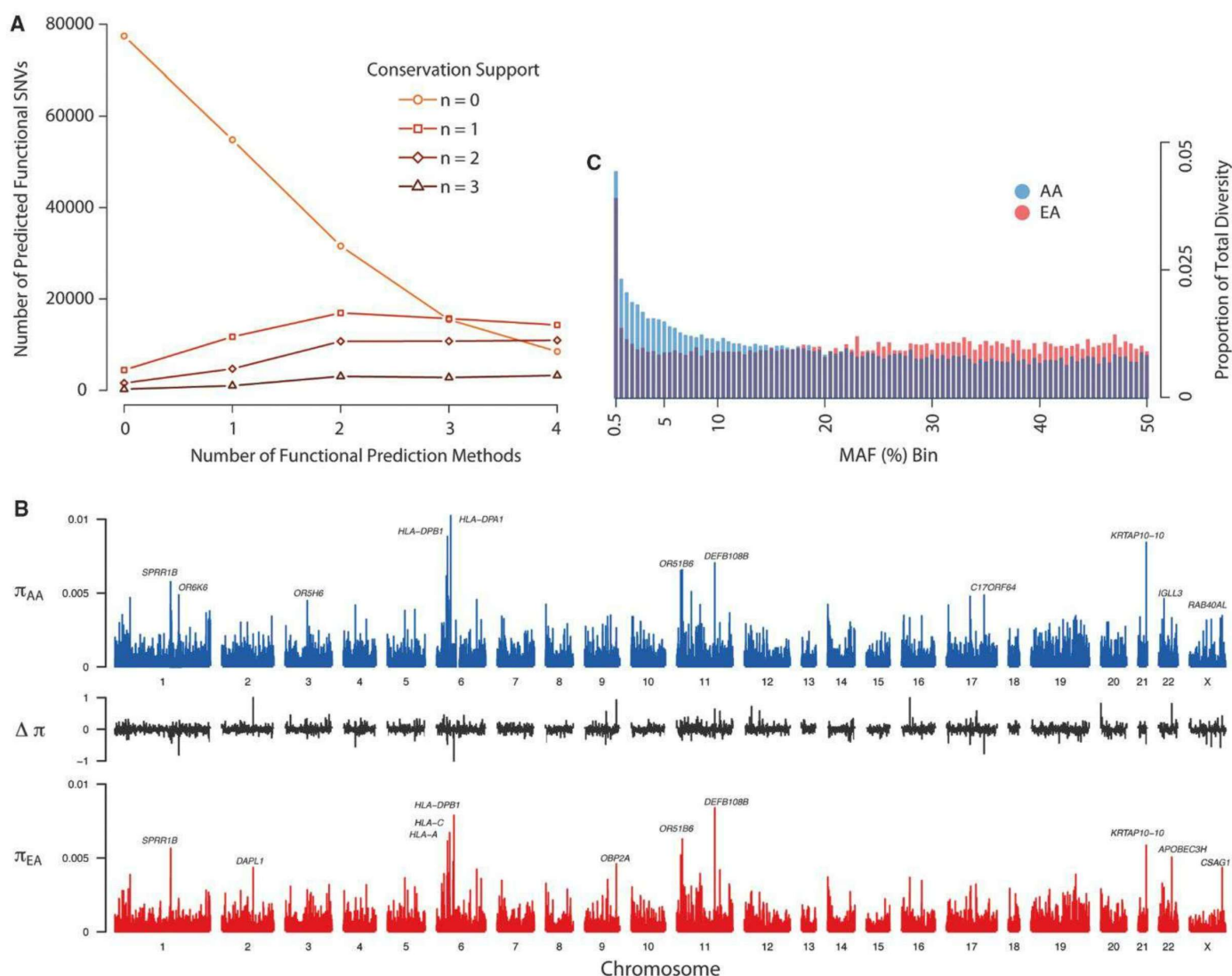


Fig. 1. Characteristics of protein-coding variation in humans. (A) Number of nonsynonymous SNVs predicted to be functionally important as a function of seven different methods (18). (B) Distributions of π across the exome in AAs (blue) and EAs (red). The value of π for each gene is shown as a vertical line.

The middle section shows the difference in diversity between EA and AA ($\Delta\pi = \pi_{EA} - \pi_{AA}$), scaled between 0 and 1. (C) Distributions of the proportion of total diversity, π , attributable to SNVs with different MAFs in the EA and AA samples. The x axis is binned in increments of 0.5%.

was 24, ranged between 0 and 761, and was significantly different (Wilcoxon-rank sum test; $P < 10^{-15}$) between AAs (median of 16, range from 0 to 566) and EAs (median of 13, range from 0 to 410). Mutational target size plays an important role in governing differences in polymorphism across loci, because gene length accounts for 76.6% of variation in the number of SNVs across genes (95% bootstrap CI = 73.9 to 79.1%; $P < 10^{-15}$).

The proportion of rare variants per base pair (bp) in each gene was higher (mean = 2.015%; 95% range = 0.621 to 4.057%) than that of common variants (0.334%; 95% range = 0.000 to 1.143%), and the average ratio of rare to common alleles per bp was ~6:1. We identified 110 genes that showed an unusually high proportion of rare variants, including six histone genes that are likely subject to greater selective constraint (table S3). The number of putatively functional variants also varied widely across genes (fig. S14B), ranging from 0 to >100, with a median of two in both EA and AA samples.

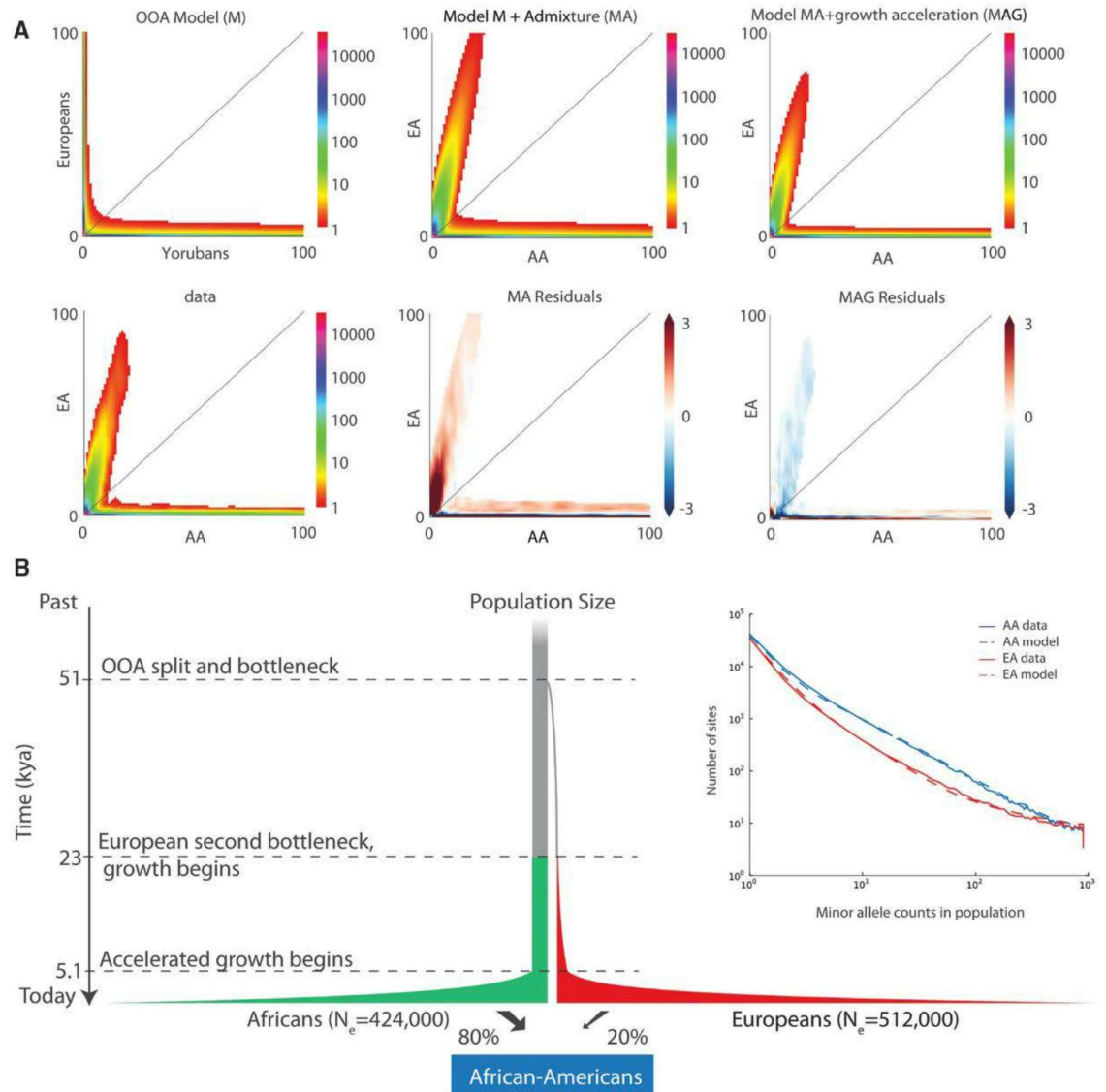
Nucleotide diversity (π) varied considerably among genes, ranging from ~0 to 1.319%

per bp (mean = 0.042%; Fig. 1B). Mean π in AAs (0.047%) was significantly higher ($P < 10^{-15}$, paired t test) than π in EAs (0.035%), and π per gene was modestly correlated ($r^2 = 63\%$; P value $< 10^{-15}$) between AAs and EAs (fig. S15). Rare variants account for 4% of total diversity, more than any other MAF bin (of width 0.5%) in both EAs and AAs (Fig. 1C). Rare and low-frequency SNVs comprise ~13 and 20% of total diversity in the EA and AA samples, respectively (Fig. 1C). In both samples, estimates of π were highest for human lymphocyte antigen (HLA) loci and other genes related to immune function, such as *DEFB108B*, and olfactory receptors (Fig. 1B). When genes were grouped into functional categories by KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway, estimates of π were highest for pathways related to immune function and olfaction and lowest for pathways involved in basic cellular processes (fig. S16).

Abundance of rare variation explained by human demographic history. The excess of rare

variation across the exome is consistent with explosive human population growth (22). To investigate this further, we used an out-of-Africa (OOA) demographic model (23) to predict the expected joint distribution of allele frequencies between EA and AA samples via a diffusion approximation (18). The OOA model, modified to account for admixture, captures prominent features of the joint frequency distribution. However, both populations contain more rare variants than predicted by this model (18) (Fig. 2), most likely because of rapid population growth in the past few thousand years that is undetectable with smaller sample sizes (fig. S9E). We revisited the demographic model from Gravel *et al.* (23), allowing for a reduced initial European expansion that is compensated for by accelerated growth starting after the split of European and Asian populations. Similarly, we introduced a phase of exponential growth in the African population starting at the same time. The resulting demographic model is an improved fit to the synonymous site-frequency spectrum (18) (Fig. 2B)

Fig. 2. Deep sequencing reveals increases of recent population size. (A) Joint SFS predicted from different demographic models (top) compared with the observed data (bottom), displaying allele counts between 0 and 100 chromosomes. The three models are (left) an OOA model without admixture derived from the 1000 Genomes data, (middle) the same model with the AA panel modeled as an 80%:20% admixture between African and European lineages, and (right) the same model further modified to account for recent growth acceleration. Anscombe residuals are displayed, with regions showing more variants than predicted by the model in blue and less in red. Bins with expected counts <1 are displayed as white in all graphs. (B) Schematic representation (not to scale) of the inferred demographic model and parameters (18). kya, thousand years ago. (Inset) Comparison of the observed SFS to that predicted by the demographic model incorporating recent accelerated growth.



and strongly supports a recent, dramatic acceleration of population growth. The maximum-likelihood time for accelerated growth was 5115 years ago (Fig. 2B).

The EA population growth, previously estimated at 0.38% per generation, is now modeled at the first step as 0.307% (SD of $\pm 0.003\%$), followed by explosive growth of 1.95% (SD $\pm 0.03\%$) over the past 5115 years. The growth in the AA sample during this same period is estimated to be 1.66% (SD $\pm 0.03\%$). The estimated standard deviations (18) are quite small, and, for data sets of this scale, it is likely that other sources of uncertainty (e.g., mutation rate or model specification) play a more important role than finite genome fluctuations. The final population sizes in this model are lower than current census sizes, and we speculate that larger sample sizes will be necessary to fully capture the signature recent growth-rate expansion imparted on patterns of DNA sequence variation.

Impact of natural selection on rare coding variation. To investigate the effect of purifying selection on nonsynonymous variants, we examined the relationship between MAFs of nonsynonymous SNVs and functional prediction scores from SIFT, Polyphen2, a likelihood ratio test statistic, and MutationTaster (18). Each prediction score showed a significant ($P < 10^{-16}$) negative correlation with MAF in the combined sample (Fig. 3A) as well as in each sample separately (18). Moreover, the proportion of predicted

deleterious changes was negatively correlated with MAF (Fig. 3B). We next mapped 31,115 nonsynonymous SNVs to known protein structures and classified them into different structural categories (Fig. 3C) (18). Nonsynonymous SNVs in all categories, except sites that contact other protein chains, showed a significant excess of rare variants compared with synonymous sites (Fig. 3C), as expected if subjected to purifying selection. The relative effect sizes show that categories of variants with direct functional importance (e.g., active sites, enrichment of 2.8%; ligand-binding residues, enrichment of 1.7%) are under stronger constraint than categories important for structural stability. The exception is residues that form side-chain hydrogen bonds, which show a 2.8% enrichment of rare variants, suggesting that hydrogen bonds make a large contribution to protein folding and stability.

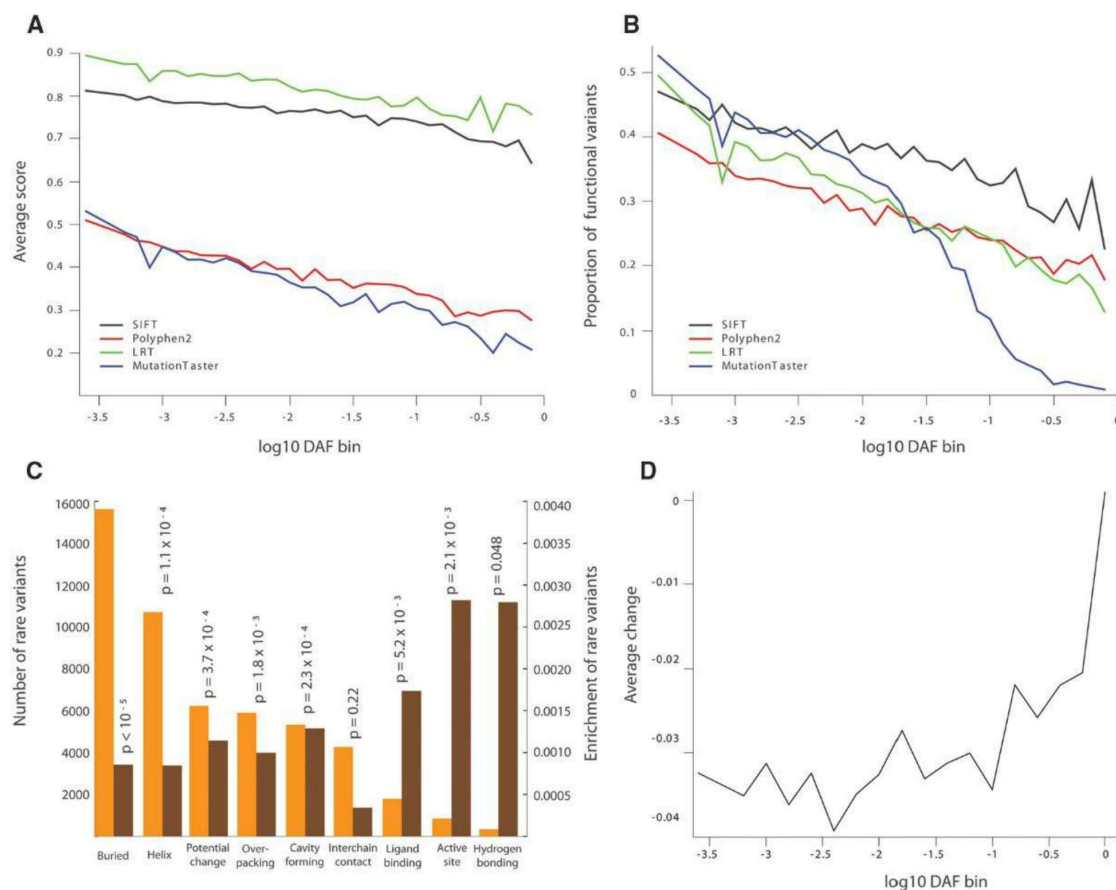
To investigate selective constraint acting on synonymous variants, we calculated the correlation between the derived allele frequency (DAF) of synonymous variants and their corresponding change in the relative adaptiveness value, or w score (24). The w score summarizes information about selective constraints on the efficiency of codon-anticodon coupling and the number of tRNA gene copies in the genome. Negative values indicate synonymous variants that may decrease translational efficiency or accuracy. We found a weak but significant positive correlation between DAF and change in w score ($r = 0.03$;

$P < 10^{-16}$), consistent with the action of purifying selection (Fig. 3D).

We examined selective sweeps by identifying genes with high ratios of divergence (human-specific lineage substitutions relative to chimp and macaque) compared with polymorphism within humans, which are predicted to increase between-species divergence and decrease within-population diversity. We identified genes in which the ratio of nonsynonymous to synonymous divergence was high relative to the ratio of nonsynonymous to synonymous SNVs (25). We also identified genes with either a high or low ratio of π in AAs relative to π in EAs and genes with diversity estimates in the bottom 20th percentile in which at least one SNV had an $F_{ST} \geq 0.3$. In total, 114 genes met one or more of these criteria (table S4). About 25% of these genes have been implicated as targets of positive selection (26). The 114 candidate selection genes were significantly enriched (false discovery rate $\leq 5\%$) for five KEGG pathways, including olfactory transduction and metabolic pathways (table S5).

Implications for disease and personal genomics. We evaluated gene-specific power of rare variant association studies in the EA and AA samples. We used Fisher's exact test, a robust approach for aggregate testing of rare variation at a locus (27), to determine the power to detect an association for each gene harboring rare causal variants with ORs of 1.5 or 5 in 400 cases and 400 controls (18). In both the EA and AA samples,

Fig. 3. Signatures of purifying selection in protein-coding SNVs. **(A)** Relationship between the evidence that a variant is functionally important and MAF for four different methods. **(B)** Relationship between the proportion of putatively functional variants and MAF for the same predictions as in (A). **(C)** Comparison of the number of rare SNVs (orange) and enrichment of rare or nonsynonymous SNVs (brown) located in different protein structural categories [P values were calculated by a permutation test (18)]. **(D)** Relationship between average change of w score of synonymous variants and DAF.



cases and controls were sampled from 1000 individuals selected to minimize any confounding effects of population stratification (fig. S17), with power calculations assuming a type I error rate of $\alpha = 0.001$. In each sample, power varies widely across loci, and $<5\%$ of genes achieve 80% power even when relatively strong effects ($OR = 5$) are modeled (Fig. 4A); when causal variants are assumed to have an OR of 1.5, no genes achieve 80% power (fig. S18). Furthermore, although the AA sample has uniformly higher power per gene relative to the EA sample (Fig. 4A), caution is warranted because this is largely a function of our modeling assumptions (18).

The mean number of SNVs per exome (homozygous nonreference and heterozygous genotypes) was 13,595, and $\sim 66\%$ (8893) of these sites were heterozygous. As expected, AAs had significantly more SNVs per exome than EAs

(15,073 versus 12,406, Mann-Whitney test, $P < 10^{-16}$), which is true for all classes of sites (Fig. 4B). Moreover, on average, each individual possessed 35 nonsense variants and was homozygous for at least one nonreference nonsense variant; 318 individuals (181 AAs and 137 EAs) were compound heterozygotes for nonsense SNVs. The mean number of novel SNVs per individual was 549 overall, but AAs had more than twice the number of novel SNVs compared with EAs (762 versus 362, respectively; $P = 1.9 \times 10^{-7}$ correcting for differences in the mean number of SNVs between populations). The fraction of overall variation that was novel in AAs was higher than in EAs (5 and 3%, respectively; $P < 10^{-16}$). Lastly, although most protein-coding variants were rare in the full AA and EA population samples, the majority of SNVs found in an average individual were common (Fig. 4C).

We next examined the distribution of functionally important variation, functionally important singletons, and the proportion of functionally important SNVs per individual (Fig. 4D) by using both conservative and more liberal criteria (18). On average, individuals possess between 318 and 580 predicted functional protein-coding SNVs depending on how functional variants are defined, with slightly more in AA than in EA individuals (Fig. 4D). The average number of predicted functional singletons per individual was more robust to the definition of functional variants, ranged from 25 to 31, and was slightly higher in AA compared to EA individuals (Fig. 4D). In both cases, however, there was more variation among individuals than between populations.

Lastly, the average proportion of predicted functional SNVs per individual varied between 2.3 and 4.2% (Fig. 4D). When the more liberal

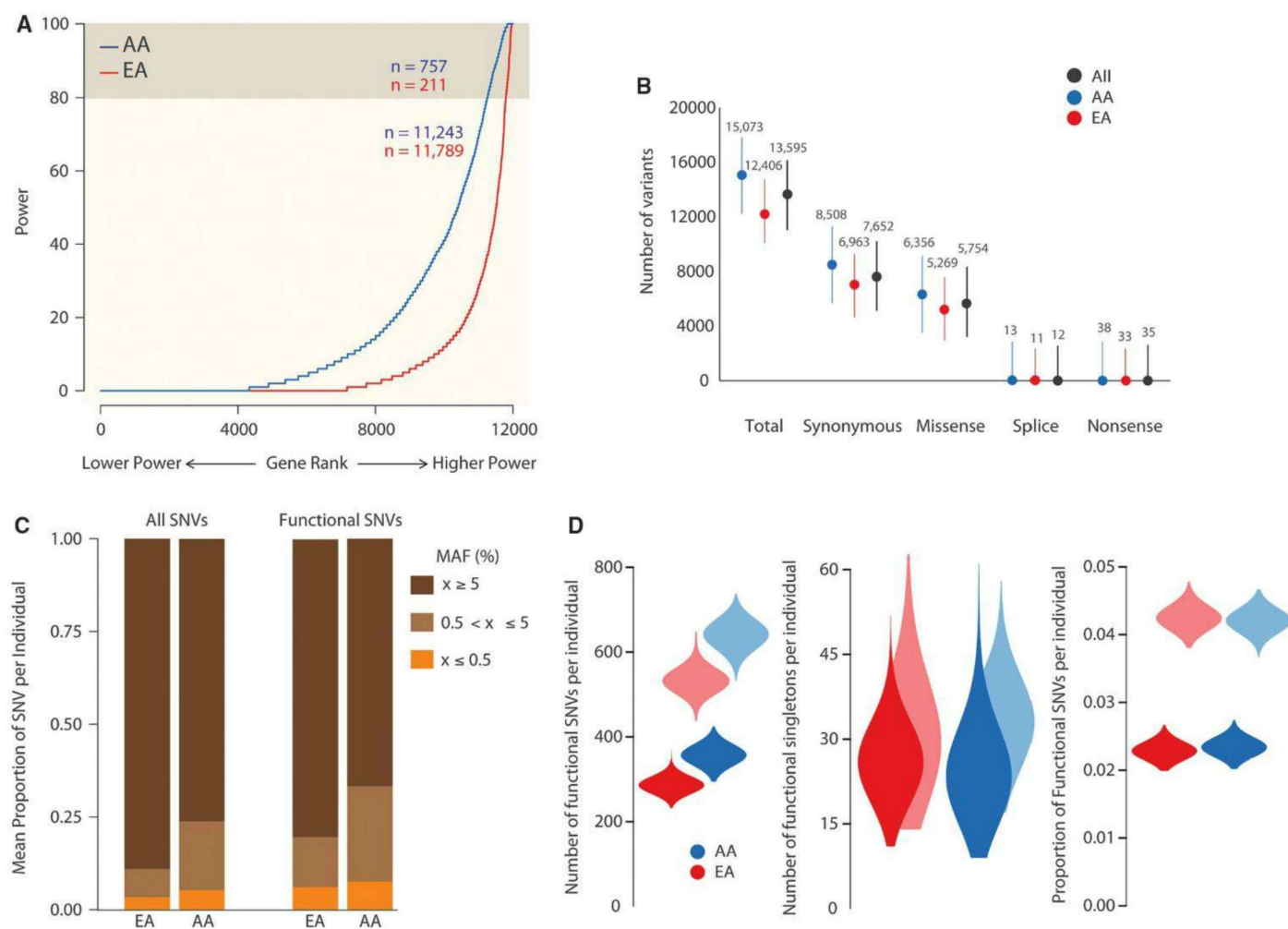


Fig. 4. Power of rare variant association mapping and personal genomics characteristics of protein-coding SNVs. **(A)** Distribution of gene-specific estimates of power to map causal rare variants across 12,000 protein-coding genes with at least three SNVs in the EA (red) or AA (blue) samples. Power varied widely across loci, and $<5\%$ of genes (beige) achieve 80% power even when relatively strong effects ($OR = 5$) are modeled. **(B)** Average number (points) and range (vertical lines) of synonymous, missense, splice site, and nonsense SNVs. **(C)** Average proportion of SNVs per individual that are rare

($MAF \leq 0.5\%$), intermediate ($0.5\% < MAF < 5\%$), or common ($MAF \geq 5\%$) in the population from which they were sampled. The proportions of rare and intermediate frequency variants per individual are significantly higher (Wilcoxon-rank sum test; $P < 10^{-15}$) for putatively functional SNVs. **(D)** Violin plots showing the distribution of number of functional SNVs, number of functional singletons, and proportion of functional SNVs per individual in the EA and AA samples. Darker and lighter shaded plots correspond to conservative and more liberal definitions of functional variation, respectively.

definition of functional SNVs was used, EA individuals have a significantly higher proportion of predicted functional SNVs compared with AA individuals (Fig. 4D; Wilcoxon-rank sum test; $P < 10^{-15}$), consistent with empirical estimates and theoretical expectations because of the lower EA effective population size (28, 29). However, when the more conservative definition was used, this pattern was reversed, and AA individuals have a significantly higher proportion of predicted functional SNVs compared with EA individuals (Fig. 4D; Wilcoxon-rank sum test; $P < 10^{-15}$). These results highlight how the definition of functional variants can influence inferences and underscore the importance of continued methodological development to robustly identify functionally important variation. Nonetheless, there was considerable rare genetic variation among individuals that is predicted to be functional, which could explain variability in disease risk and adverse drug response.

Conclusion. Our results have several important implications for human disease gene mapping and personal genomics. In particular, the vast majority of protein-coding variation is evolutionarily recent, rare, and enriched for deleterious alleles. Thus, rare variation likely makes an important contribution to human phenotypic variation and disease susceptibility. However, detecting the effects of rare variants requires very large sample sizes, because the power to detect an association is low for most human genes. Accounting for the SFS on a gene-by-gene basis should facilitate the development of more powerful association tests. Additionally, because most rare SNVs are population-specific, replication of disease associations across populations may be challenging. Lastly, as whole-genome sequencing at high coverage becomes increasingly feasible, statistical and experimental

methods that accurately identify functionally important protein-coding and regulatory variation are needed to empower association studies, identify variants causally related to disease, and provide clinically actionable information.

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(LungGO), and RC2 HL-102924 (Women's Health Initiative Exome Sequencing Project, WHISP). The exome sequencing was performed through NHLBI grants RC2 HL-102925 (BroadGO) and RC2 HL-102926 (SeattleGO). Filtered sets of annotated variants and their allele frequencies are available at <http://evs.gs.washington.edu/EVS/> and have been deposited in dbSNP (www.ncbi.nlm.nih.gov/snp; local batch ID, ESP2500). Genotypes and phenotypes from a large subset of individuals are also available via dbGaP (www.ncbi.nlm.nih.gov/gap) using the following accession information: NHLBI GO-ESP: Women's Health Initiative Exome Sequencing Project (WHI) – WHISP, WHISP_Subject_Phenotypes, ph002246.v2.p2, phs000281.v2.p2; NHLBI GO-ESP: Heart Cohorts Exome Sequencing Project (JHS), ESP_HeartGO_JHS_LDandEOMI_Subject_Phenotypes, ph002539.v1.p1, phs000402.v1.p1; NHLBI GO-ESP: Heart Cohorts Exome Sequencing Project (FHS), HeartGO_FHS_LDandEOMI_PhenotypeDataFile, ph002476.v1.p1, phs000401.v1.p1; NHLBI GO-ESP: Heart Cohorts Exome Sequencing Project (CHS), HeartGO_CHS_LD_PhenotypeDataFile, ph002536.v1.p1, phs000400.v1.p1; NHLBI GO-ESP: Heart Cohorts Exome Sequencing Project (ARIC), ESP_ARIC_LDandEOMI_Sample, ph002466.v1.p1, phs000398.v1.p1; NHLBI GO-ESP: Lung Cohorts Exome Sequencing Project (Cystic Fibrosis), ESP_LungGO_CF_PA_Culture_Data, ph002227.v1.p1, phs000254.v1.p1; NHLBI GO-ESP: Early-Onset Myocardial Infarction (Broad EOMI), ESP_Broad_EOMI_Subject_Phenotypes, ph001437.v1.p1, phs000279.v1.p1; NHLBI GO-ESP: Lung Cohorts Exome Sequencing Project (Pulmonary Arterial Hypertension), PAH_Subject_Phenotypes_Baseline_Measures, ph002277.v1.p1, phs000290.v1.p1; NHLBI GO-ESP: Lung Cohorts Exome Sequencing Project (Lung Health Study of Chronic Obstructive Pulmonary Disease), LHS_COPD_Subject_Phenotypes_Baseline_Measures, ph002272.v1.p1, phs000291.v1.p1. C.D.B. is on the scientific advisory board for Personalis, Incorporated; Mubadala Medical Holding Company; 23andme "Roots into the future" project; and Ancestry.com. M.J.R. owns stock in Illumina.

Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1219240/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S19
Tables S1 to S7
References (30–47)

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REPORTS

Interferometric Identification of a Pre-Brown Dwarf

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It is not known whether brown dwarfs [stellar-like objects with masses less than the hydrogen-burning limit, 0.075 solar mass ($M_{\odot}$)] are formed in the same way as solar-type stars or by some other process. Here we report the clear-cut identification of a self-gravitating condensation of gas and dust with a mass in the brown-dwarf regime, made through millimeter interferometric observations. The level of thermal millimeter continuum emission detected from this object indicates a mass ~ 0.02 to $0.03 M_{\odot}$, whereas the small radius, <460 astronomical units, and narrow spectral lines imply a dynamical mass of 0.015 to $0.02 M_{\odot}$. The identification of such a pre-brown dwarf core supports models according to which brown dwarfs are formed in the same manner as hydrogen-burning stars.

Brown dwarfs, defined as stellar-like objects with masses less than the hydrogen-burning limit $M_{BD} = 0.075$ solar mass

($M_{\odot}$) (1), were first discovered in 1995 (2, 3). They are now known to be almost as numerous as hydrogen-burning stars (4–6), but their formation

mechanism remains a matter for debate (6, 7). Either brown dwarfs form as a by-product of the formation process of hydrogen-burning stars, or they form just like normal stars (7), from the collapse of self-gravitating condensations of gas and dust called prestellar cores (8). The models in the former category include models of multiple star formation, where the lowest-mass member is ejected before accreting too much mass (9, 10);

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models of circumstellar disk fragmentation (11, 12); and models in which more-massive prestellar cores are disrupted by nearby massive star formation into low-mass cores, which are then triggered into collapse (13). The types of models we test here are those in which brown dwarfs form like solar-mass stars, but are just less massive, as in the gravoturbulent fragmentation scenario (14, 15). In this theoretical scenario, gas cores with a wide range of masses are formed from material compressed by shocks resulting from supersonic interstellar turbulence. At low masses, this includes both gravitationally bound and unbound cores. A fraction of the low-mass cores produced by turbulence are dense enough to be gravitationally unstable to collapse: Their masses exceed the local Jeans or Bonnor-Ebert mass and are comparable to their virial mass. Indeed, turbulence can locally generate sufficient compression and background pressure ($P_{\text{back}}/k_B > P_{\text{BD},10K}/k_B \approx 2.4 \times 10^7 \text{ K cm}^{-3}$) (k_B , Boltzmann constant), that the corresponding critical Bonnor-Ebert mass, $M_{\text{BE}} = 0.12 M_{\odot} \times (T/10 \text{ K})^2 \times (P_{\text{back}}/10^7 \text{ K cm}^{-3})^{-1/2}$ (T , temperature), which has a typical value of $\sim 1 M_{\odot}$ in molecular clouds (16), becomes smaller than the maximum brown-dwarf mass $M_{\text{BD}} = 0.075 M_{\odot}$. This scenario predicts a large number of pre-brown dwarfs, which can be approximately described as compact Bonnor-Ebert isothermal spheres of mass $M_{\text{BE}} < M_{\text{BD}}$ and radius $R_{\text{BE}} < R_{\text{BD}} \approx 700$ astronomical units (AU) (17), but no good example of such a pre-brown dwarf core has previously been confirmed observationally to be sufficiently compact to be self-gravitating.

Nearby, high-pressure cluster-forming regions such as L1688 in Ophiuchus [120 to 140 parsecs (pc) from the Sun (18, 19)] are the best places to search for pre-brown dwarfs. Following up on an early DCO⁺ search (20), deep dust continuum observations with the SCUBA camera on the James Clerk Maxwell Telescope (JCMT) led to the detection of a candidate pre-brown dwarf in L1688 as an unresolved 850- μm point source with flux density ($S_{850\mu\text{m}} = 39 \pm 5 \text{ mJy/15'' beam}$) (21). This candidate, called Oph B-11, is located in a region of relatively high visual extinction ($A_V \sim 30$ mag based on data from the 2MASS survey catalog) between the dense clumps Oph B1 and Oph B2 of L1688 [(22) and fig. S1].

We used the Institut de Radioastronomie Millimétrique (IRAM) Plateau de Bure interferometer (PdBI) in CD configuration to observe Oph B-11 at high spatial resolution at 3.2 mm, for a total of four interferometer tracks between March 2006 and April 2011. We obtained a robust detection of the source in both the continuum at 3.2 mm (Fig. 1) and the $\text{N}_2\text{H}^+(1-0)$ line (Fig. 2 and fig. S2). The 3.2-mm continuum PdBI source is located less than $2''$ away from the previously detected SCUBA position (21) (which has an uncertainty of $\pm 3''$). It has a peak flux density $S_{3.2\text{mm, peak}} \approx 0.32 \pm 0.05 \text{ mJy/7.8''} \times 2.8''$ beam ($>6\sigma$ detection) and an integrated flux density $S_{3.2\text{mm, tot}} = 0.4 \pm 0.1 \text{ mJy}$. The probability of chance coincidence with an extragalactic object

is $<0.05\%$ (see the supplementary materials). The source is pointlike at the PdBI resolution. Fitting a spherical Bonnor-Ebert core model (23) to the

interferometric visibilities in the Fourier plane yields a best-fit value of $1.0'' \pm 1.8''$ for the outer angular radius. This corresponds to a best-fit core

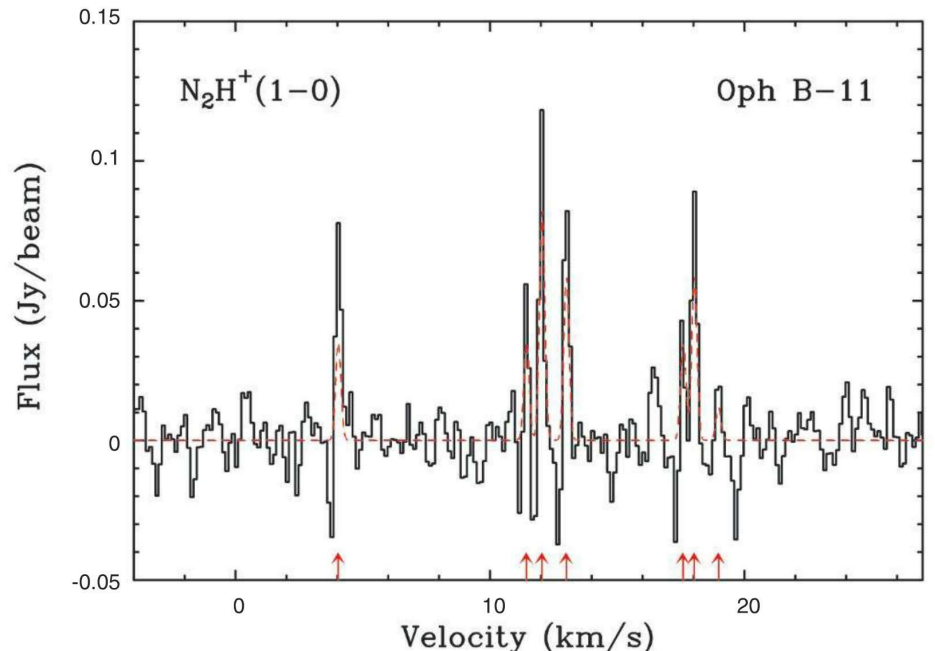
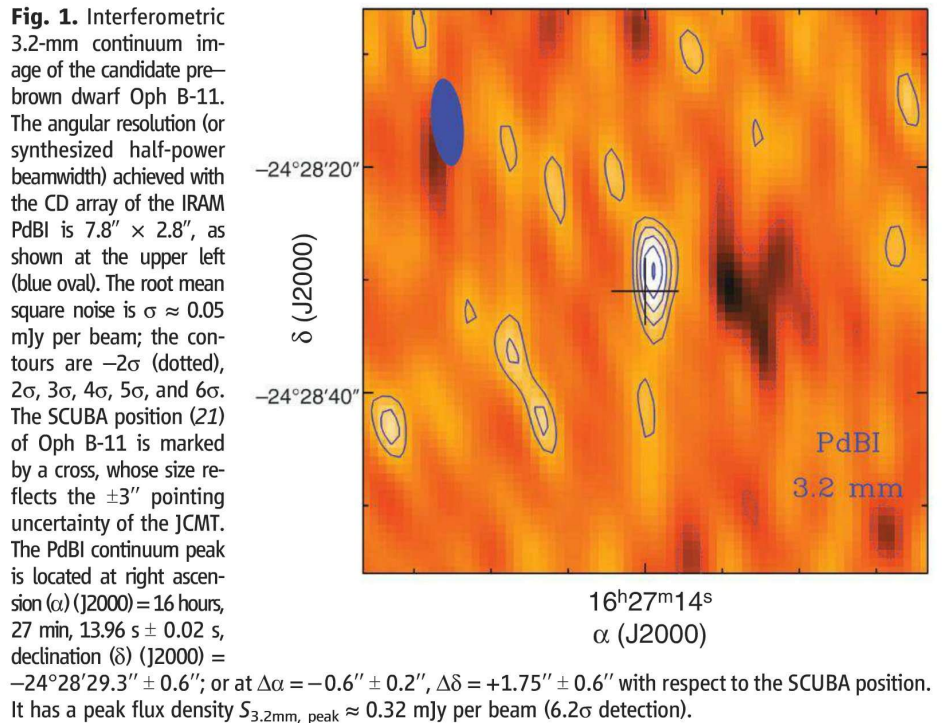


Fig. 2. Spectrum of Oph B-11 in the $\text{N}_2\text{H}^+(1-0)$ line multiplet. The data (black curve) correspond to the beam-averaged $\text{N}_2\text{H}^+(1-0)$ spectrum observed with the D array of the IRAM PdBI at the position of the 3.2-mm continuum peak of Oph B-11 (Fig. 1). The x axis represents V_{LSR} , assuming a rest frequency of 93176.265 MHz for the isolated component of the line multiplet $\text{N}_2\text{H}^+(101-012)$. A Gaussian fit to the $\text{N}_2\text{H}^+(1-0)$ multiplet (superimposed as a dashed red curve) yields a full-width-at-half-maximum (FWHM) linewidth $\Delta v_{\text{obs}} = 0.21 \pm 0.03 \text{ km/s}$, whereas the velocity resolution is $\Delta v_{\text{res}} = 0.125 \text{ km/s}$. The expected positions of the seven components of the $\text{N}_2\text{H}^+(1-0)$ line multiplet are marked by red arrows. The fit implies a deconvolved FWHM linewidth $\Delta v_{\text{int}} = 0.17 \pm 0.04 \text{ km/s}$, corresponding to a nonthermal velocity dispersion $\sigma_{\text{NT}} = 0.05 \pm 0.02 \text{ km/s}$. The systemic velocity of the source is $V_{\text{LSR}} = 4.03 \pm 0.04 \text{ km/s}$, similar to that of other objects in the dense clumps Oph B1 and Oph B2 of the Ophiuchus protocluster (22).

radius $R_{\text{core}} \sim 140$ AU if we adopt a distance $d = 140$ pc for the Ophiuchus cloud (19).

The interferometric visibility curve sets an upper limit of $3.3''$ to the outer angular radius at the 90% confidence level (Fig. 3). This constrains the outer radius of the Bonnor-Ebert core model to be $R_{\text{core}} < 460$ AU. The presence of an extended power-law envelope around the compact core is ruled out by the shape of the visibility curve (Fig. 3) and the low value of the $850\text{-}\mu\text{m}$ flux density measured in a $15''$ beam at the JCMT (21).

We know that the Oph B-11 core is starless because it was not detected in deep mid-infrared observations of L1688 with the Spitzer space telescope (24). It also remained undetected at both 70 and $100\text{ }\mu\text{m}$ in recent Herschel space observatory observations of L1688 made as part of the Herschel Gould Belt survey (25).

Despite the high level of background emission from the L1688 cloud, the Herschel $70\text{-}\mu\text{m}$ data set a 3σ upper limit of 85 mJy on the flux density of Oph B-11 at $70\text{ }\mu\text{m}$, which is significantly lower than the $70\text{-}\mu\text{m}$ flux density of ~ 200 to 300 mJy expected for a first hydrostatic protostellar core at the distance of Ophiuchus (26, 27). Therefore, it is safe to conclude that there is no protostellar object at the center of Oph B-11. The available spectral energy distribution of Oph B-11 is consistent with emission from cold ($T \lesssim 10\text{ K}$) dust (Fig. 4). Furthermore, the ambient radiation field in the L1688 cloud is relatively well constrained (28), and radiative transfer calculations (29) confirm that the core temperature should be as low as ~ 8.5 to 10 K , given the high visual extinction $A_V \sim 30$ mag observed in the immediate vicinity of Oph B-11.

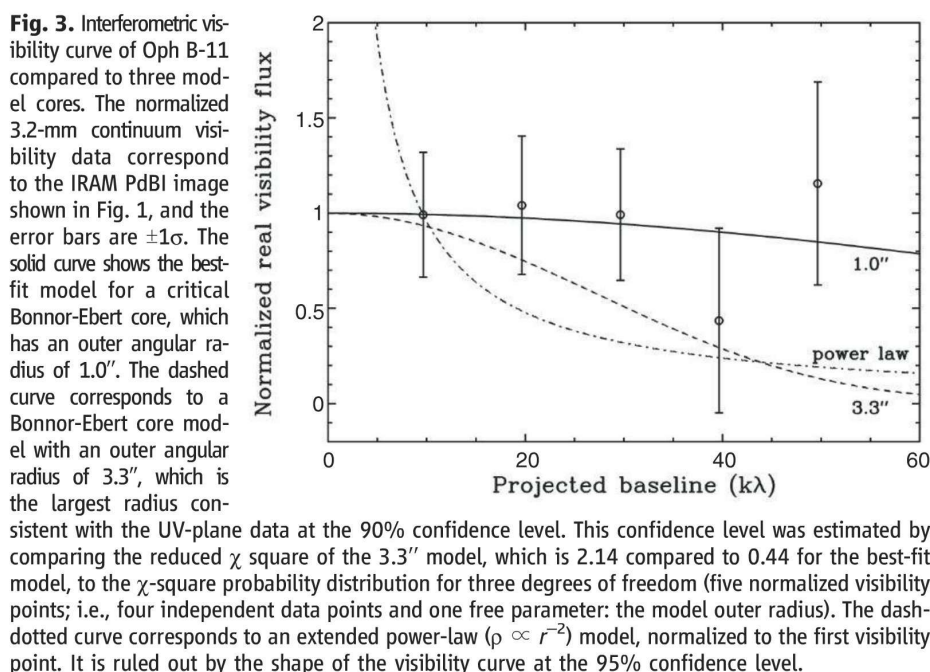


Fig. 3. Interferometric visibility curve of Oph B-11 compared to three model cores. The normalized 3.2-mm continuum visibility data correspond to the IRAM PdBI image shown in Fig. 1, and the error bars are $\pm 1\sigma$. The solid curve shows the best-fit model for a critical Bonnor-Ebert core, which has an outer angular radius of $1.0''$. The dashed curve corresponds to a Bonnor-Ebert core model with an outer angular radius of $3.3''$, which is the largest radius consistent with the UV-plane data at the 90% confidence level. This confidence level was estimated by comparing the reduced χ square of the $3.3''$ model, which is 2.14 compared to 0.44 for the best-fit model, to the χ -square probability distribution for three degrees of freedom (five normalized visibility points; i.e., four independent data points and one free parameter: the model outer radius). The dash-dotted curve corresponds to an extended power-law ($\rho \propto r^{-2}$) model, normalized to the first visibility point. It is ruled out by the shape of the visibility curve at the 95% confidence level.

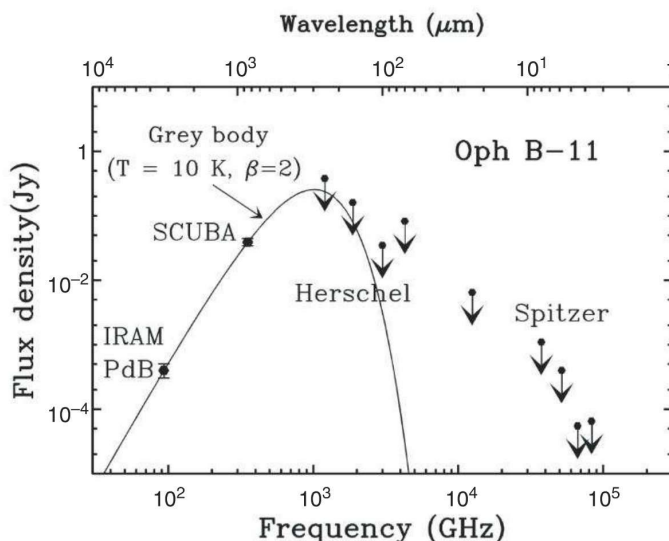


Fig. 4. Spectral energy distribution of the Oph B-11 core, including the present IRAM PdBI 3.2-mm detection, the original SCUBA $850\text{-}\mu\text{m}$ detection (21), and 3σ upper limits at other wavelengths based on far-infrared data from the Herschel Gould Belt survey (25) and mid-/near-infrared data from Spitzer (24). Error bars on detections are $\pm 1\sigma$. The solid curve shows a 10 K gray body, consistent with all observational constraints.

Assuming a typical dust opacity law for starless cores (30, 31) (i.e., an opacity per unit of gas+dust mass column density $\kappa_{850\mu} \sim 0.01\text{ cm}^2\text{ g}^{-1}$ and a dust emissivity index $\beta = 2$), the observed $850\text{-}\mu\text{m}$ (21) and 3.2-mm flux densities of Oph B-11 correspond to a mass of gas and dust $M_{\text{obs}} \sim 0.02$ to $0.03 M_{\odot}$ at $d = 140$ pc for a dust temperature in the range of 8.5 to 10 K (see the supplementary materials for a discussion of the uncertainties). Adopting the upper limit of 460 AU to the core radius, the lower limits to the mean density and mean column density are estimated to be $\langle n_{\text{H}_2} \rangle \gtrsim 7.5 \times 10^6\text{ cm}^{-3}$ and $\langle N_{\text{H}_2} \rangle \gtrsim 6.9 \times 10^{22}\text{ cm}^{-2}$, respectively. Therefore, Oph B-11 has a minimum mean column-density contrast of a factor ≥ 2.3 over the local background, which has a typical column density $\sim 3 \times 10^{22}\text{ H}_2\text{ cm}^{-2}$ (fig. S1).

The linewidth measured for the $\text{N}_2\text{H}^+(1-0)$ multiplet is extremely narrow (Fig. 2), indicating that the total (thermal + nonthermal) one-dimensional velocity dispersion σ_{tot} within the core is nearly thermal and is comparable to the isothermal sound speed ($c_s \lesssim 0.2\text{ km/s}$ for a gas temperature $T \lesssim 10\text{ K}$). For a truncated $\rho \propto r^{-2}$ density distribution, the nominal virial mass or dynamical mass of Oph B-11 is estimated to be $M_{\text{vir}} = 3R_{\text{core}}\sigma_{\text{tot}}^2/G = 0.014$ to $0.017 M_{\odot}$ for the best-fit core radius and $T = 8.5$ to 10 K . If the core radius is as large as the upper limit of 460 AU, then the virial mass reaches an upper limit of $<0.056 M_{\odot}$. The virial mass ratio $\alpha_{\text{vir}} \equiv M_{\text{vir}}/M_{\text{obs}}$ of the Oph B-11 core thus has a nominal value of 0.5 to 0.9 and an upper limit of 2.8 . On the theoretical side (32), $\alpha_{\text{vir}} \gg 2$ when self-gravity is unimportant, $\alpha_{\text{vir}} \lesssim 2$ for gravitationally bound objects, and $\alpha_{\text{vir}} \lesssim 1$ for strongly self-gravitating objects, in which gravitational energy dominates over kinetic energy. Both the derived value of α_{vir} and the high column-density contrast strongly suggest that Oph B-11 is gravitationally bound. Because the present values of M_{obs} and M_{vir} are both smaller than the brown dwarf limit of $0.075 M_{\odot}$ and the estimated final mass of the core is also substellar (supplementary materials), we conclude that Oph B-11 is a pre-brown dwarf.

Our interferometric observations thus demonstrate that pre-brown dwarfs do exist and therefore tend to support models such as the gravoturbulent fragmentation picture (15, 17). However, they do not yet prove that pre-brown dwarfs are the main channel of brown dwarf formation. Although Oph B-11 itself is unlikely to have been ejected from a disk (supplementary materials), our results do not rule out the possibility that some brown dwarfs form by disk fragmentation and/or ejection (9–12).

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Supplementary Materials

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Supplementary Text

Figs. S1 and S2

References (33–48)

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Heralded Entanglement Between Widely Separated Atoms

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Entanglement is the essential feature of quantum mechanics. Notably, observers of two or more entangled particles will find correlations in their measurement results that cannot be explained by classical statistics. To make it a useful resource, particularly for scalable long-distance quantum communication, the heralded generation of entanglement between distant massive quantum systems is necessary. We report on the creation and analysis of heralded entanglement between spins of two single rubidium-87 atoms trapped independently 20 meters apart. Our results illustrate the viability of an integral resource for quantum information science, as well as for fundamental tests of quantum mechanics.

Entanglement between distant stationary quantum systems will be a key resource for future applications in the field of long-distance quantum communication, such as quantum repeaters (1) and quantum networks (2). At the same time, it is an essential ingredient for new experiments on the foundations of physics, in particular for a first loophole-free test of Bell's inequality (3–5). Central to all these applications is the heralded generation of entanglement, i.e., a signal is provided once an entangled pair is successfully prepared.

Until now, (unheralded) entanglement between separated massive quantum objects has been achieved for various systems (6, 7), even over a distance of 21 m (8). Heralded entanglement has been demonstrated with cold atomic ensembles (9, 10), single trapped ions (11, 12), and diamond crystals (13), albeit over short distances in a single setup only. For the realization of

heralded entanglement over long distances, single neutral atoms are promising candidates. In view of future applications, several important milestones have already been demonstrated for such systems: manipulation of atomic quantum registers (14), storage of quantum information (8, 15–17), fast and highly efficient state analysis (18), deterministic quantum gates between nearby trapped atoms via Rydberg blockade (19, 20), and distribution of light-matter entanglement over large distances (21).

We report on the preparation and analysis of heralded entanglement between two single ⁸⁷Rb atoms over a distance of 20 m via entanglement swapping (22). The scheme starts with entangling the spin of each of the two atoms with the polarization state of a spontaneously emitted photon (23). The photons are guided to a Bell state measurement (BSM) setup where the two-photon polarization state is projected onto an entangled state, thereby providing the heralding signal. In a final step, we evaluate the entanglement between the atomic spins.

Our experimental arrangement (Fig. 1A) consists of two independently operated experiments, here called trap 1 and trap 2 (24), which

are situated in two laboratories and equipped with their own laser and control systems. In each experiment, we load a single ⁸⁷Rb atom into an optical dipole trap (25). The typical lifetime of a single trapped atom is 5 to 10 s, limited mainly by heating during the experimental process and collisions with background gas. Photons emitted by the atoms are coupled into single-mode optical fibers and guided to the BSM arrangement next to trap 1. The lengths of the optical fibers from trap 1 and trap 2 to the BSM are 5 and 30 m, respectively. To compensate for polarization drifts induced by temperature changes and mechanical stress in the 30-m fiber, an automatic polarization stabilization (21) is used. The interferometric BSM arrangement consists of a 50-50 single-mode fiber beam splitter (BS) with polarizing beam splitters (PBSs) in each of the output ports. Additional half- and quarterwave plates allow us to select the measurement basis for the BSM and the atom-photon entanglement measurements. Finally, photons are detected by four avalanche photodiodes (APDs).

First, we verify atom-photon entanglement in each experiment separately. The generation of an entangled atom-photon state starts by preparing the atom in the initial state $5^2S_{1/2}, |F=1, m_F=0\rangle$ (Fig. 1B) via optical pumping. Then the atom is excited to the state $5^2P_{3/2}, |F'=0, m_{F'}=0\rangle$ by a short optical pulse (full width at half-maximum pulse length = 21 ns). In the following spontaneous decay, the polarization of a single photon emitted into the collection optics (defining the quantization axis z) is entangled with the atomic spin (23), yielding the state

$$\begin{aligned}
 |\Psi\rangle_{\text{AP}} &= \frac{1}{\sqrt{2}}(|\downarrow_z\rangle|L\rangle + |\uparrow_z\rangle|R\rangle) \\
 &= \frac{1}{\sqrt{2}}(|\downarrow_x\rangle|V\rangle + |\uparrow_x\rangle|H\rangle)
 \end{aligned}$$

where $|L\rangle$, $|R\rangle$ denote the left- and right-circular and $|H\rangle$, $|V\rangle$ the horizontal and vertical linear

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polarization states of the photon. The atomic qubit is defined by the Zeeman states $|m_F = +1\rangle$ and $|m_F = -1\rangle$ of the ground level $5^2S_{1/2}$, $F = 1$, which we associate with spin orientations $|\uparrow\rangle_z$ and $|\downarrow\rangle_z$, respectively. Preparation and excitation of the atom are repeated until a photon is detected. Taking into account additional cooling periods required to counteract heating of the atom, the preparation and excitation of the atom can be performed 50×10^3 times per second. The overall efficiency for detecting the photon after an excitation in trap 1 (trap 2) is $\eta_1 = 0.9 \times 10^{-3}$ ($\eta_2 = 1.25 \times 10^{-3}$). These numbers include the excitation probability, the collection and coupling efficiencies as well as losses in the optics, and also the quantum efficiency of the photodetectors. Polarization analysis of the single photons is per-

formed with the BSM arrangement, which also serves to monitor fluorescence of the atom inside the trap.

To evaluate atom-photon entanglement, conditioned on the detection of the emitted photon, the internal spin state of the atom is read out (23). The detection process consists of a Zeeman state-selective stimulated Raman adiabatic passage (STIRAP) (26) with subsequent hyperfine state detection. This process can be considered as a projection of the atom onto the state $\cos(\gamma)|\uparrow\rangle_x + \sin(\gamma)|\downarrow\rangle_x$, where γ is the angle of linear polarization of the STIRAP pulse defining the measurement basis (the angle of the corresponding direction of the atomic spin is 2γ). In atom-photon correlation measurements, we register event numbers $N_{SS'}^{(\gamma,\delta)}$, where $S, S' \in \{|\uparrow\rangle, |\downarrow\rangle\}$

are the eigenstates of the spin of the atom and the photon along their respective measurement directions defined by γ and δ . Figure 2 shows the resulting correlations between atomic spin and photon polarization measurements for both traps separately. In these measurements, the photon was detected in H/V basis ($\delta = 0^\circ$) and in $\pm 45^\circ$ basis ($\delta = 45^\circ$), while the atomic measurement angles α (trap 1) and β (trap 2) were varied between 0° and 180° . The visibilities $V^{(6)}$ of the correlation curves obtained by least-squares fits are $V_1^{(0^\circ)} = 0.869 \pm 0.006$, $V_1^{(45^\circ)} = 0.900 \pm 0.006$ for trap 1, and $V_2^{(0^\circ)} = 0.895 \pm 0.004$, $V_2^{(45^\circ)} = 0.901 \pm 0.005$ for trap 2, where the given errors are the expected statistical 1σ deviations. These high visibilities, limited mainly by the quality of the atomic state read-out, demonstrate that atom-photon entanglement is reliably generated and detected with high fidelity in both traps.

The second crucial condition for preparing a highly entangled state of two trapped atoms is a high-fidelity Bell state measurement of the photons, i.e., projecting them onto maximally entangled states. We use interferometric Bell state analysis based on the Hong-Ou-Mandel effect (27). This two-photon detection scheme does not require interferometric stability on a wavelength scale, thereby relaxing the experimental requirements for long-distance quantum communication. In general, at a beam splitter, bunching (antibunching) of two photons in a symmetric (antisymmetric) state enables one to identify Bell states. In our case, a coincidence in detectors H_1V_1 or H_2V_2 (Fig. 1A) signals projection of the photons onto the state $|\Psi^+\rangle_{\text{ph}} = \frac{1}{\sqrt{2}}(|H\rangle|V\rangle + |V\rangle|H\rangle)$, and the coincidences H_1V_2 or H_2V_1 indicate projection onto the state $|\Psi^-\rangle_{\text{ph}} = \frac{1}{\sqrt{2}}(|H\rangle|V\rangle - |V\rangle|H\rangle)$, respectively. The other two symmetric Bell states $|\Phi^\pm\rangle_{\text{ph}} = \frac{1}{\sqrt{2}}(|H\rangle|H\rangle \pm |V\rangle|V\rangle)$ give yet a different result but cannot be distinguished from each other (28–30). Thus, by detecting one of the four coincidences mentioned above, we project the incoming photons unambiguously on a Bell state, thereby heralding the generation of entanglement between the separated atoms.

The visibility of the two-photon interference, which determines the fidelity of the Bell state measurement, depends crucially on temporal, spatial, and spectral indistinguishability of the arriving photons. Experimentally, the temporal overlap is achieved by synchronizing the two excitation procedures in trap 1 and trap 2 to better than 500 ps, which is far below the lifetime of the excited atomic state of 26.2 ns, and by exactly matching the shapes of the interfering wave packets (Fig. 1C). The single-mode fiber beam splitter guarantees spatial mode overlap of unity. Frequency differences of the emitted photons are minimized by zeroing all relevant fields (31). Further reduction of the fidelity of the Bell state measurement arises from two-photon emission by a single atom due to off-resonant excitation of the atom to the $5^2P_{3/2}$, $F' = 1$ level (see Fig. 1B and fig. S1) if the first photon is emitted already within the duration of the excitation pulse. However, owing to the

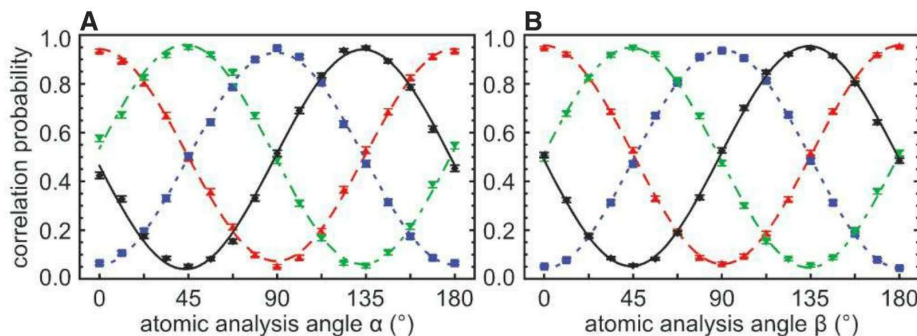
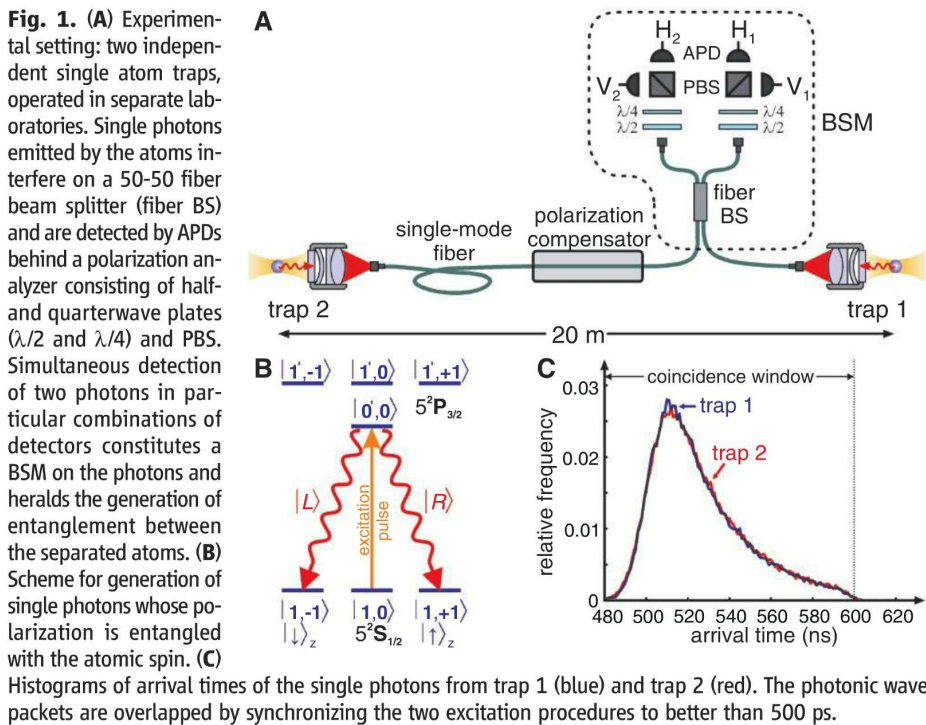


Fig. 2. Atom-photon correlations for trap 1 (A) and trap 2 (B). The graphs show the measured correlation probabilities $\frac{1}{N}(N_{\uparrow\uparrow}^{(\gamma,0^\circ)} + N_{\downarrow\downarrow}^{(\gamma,0^\circ)})$ (red triangles with dashed lines), $\frac{1}{N}(N_{\uparrow\uparrow}^{(\gamma,45^\circ)} + N_{\downarrow\downarrow}^{(\gamma,45^\circ)})$ (green inverted triangles with dotted lines), and the anticorrelation probabilities $\frac{1}{N}(N_{\uparrow\downarrow}^{(\gamma,0^\circ)} + N_{\downarrow\uparrow}^{(\gamma,0^\circ)})$ (blue squares with dotted lines), $\frac{1}{N}(N_{\uparrow\downarrow}^{(\gamma,45^\circ)} + N_{\downarrow\uparrow}^{(\gamma,45^\circ)})$ (black diamonds with solid lines), $\gamma \in \{\alpha, \beta\}$ as a function of the respective atomic analysis angle. Each point is deduced from $N = 1200$ to 2400 events, where N is the sum over the four possible measurement outcomes.

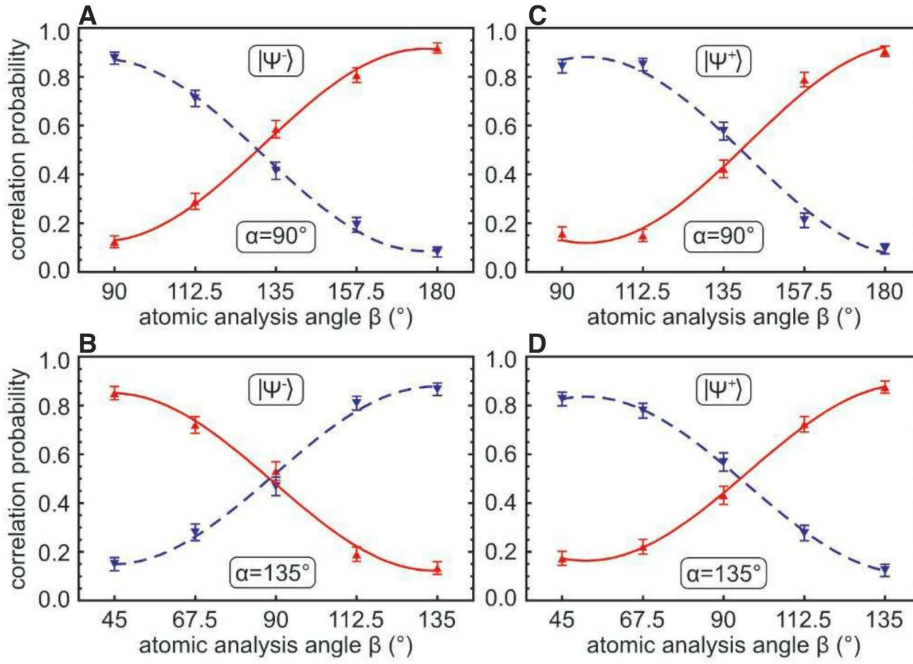


Fig. 3. Atom-atom correlations obtained after Bell state projection of the photons onto state $|\Psi^- \rangle_{\text{ph}}$ (A and B) and $|\Psi^+ \rangle_{\text{ph}}$ (C and D), respectively. The measured correlation probabilities $\frac{1}{N}(N_{\uparrow\uparrow}^{(\alpha,\beta)} + N_{\downarrow\downarrow}^{(\alpha,\beta)})$ (red triangles with solid lines), and the anticorrelation probabilities $\frac{1}{N}(N_{\uparrow\downarrow}^{(\alpha,\beta)} + N_{\downarrow\uparrow}^{(\alpha,\beta)})$ (blue inverted triangles with dashed lines) are shown for two complementary measurement bases. Each point is deduced from $N = 170$ to 190 atom-atom events. The overall number of events in this measurement is 3637, acquired within about 107 hours.

structure of the involved atomic levels, with a probability of 78.1% a polarization-entangled state $\frac{1}{\sqrt{2}}(|H\rangle_1|H\rangle_2 + |V\rangle_1|V\rangle_2)$ of the two consecutively emitted photons is formed (32). These events are registered as coincidences H_1H_2 and V_1V_2 and do not herald projection onto a Bell state. Reduction of the fidelity is therefore due to the remaining two-photon emissions and to dark counts of the detectors. On the basis of additional calibration measurements, we estimate a fidelity of the Bell state projection of at least 92% (32).

By combining all methods described above, we can generate and characterize entanglement between two distant atoms. In each of the two experiments, a single atom is captured and the atom-photon entangling sequences are repeated until two photons are detected within a time window of 120 ns in the BSM arrangement. With a coincidence probability of 0.54×10^{-6} and a repetition rate of 50 kHz, and by taking into account the fraction of time when an atom was present in each of the traps of 0.35, we arrive at an atom-atom entanglement rate of about $1/106 \text{ s}^{-1}$. A valid twofold detection, i.e., registration of $|\Psi^\pm \rangle_{\text{ph}}$, heralds projection of the atoms onto the state $|\Psi^\pm \rangle_{\text{AA}} = \frac{1}{\sqrt{2}}(|\uparrow\rangle_x|\downarrow\rangle_x \pm |\downarrow\rangle_x|\uparrow\rangle_x)$. Subsequently, measurements of the atomic states are performed 1.2 μs (trap 1) and 0.95 μs (trap 2) after the coincidence detection (fig. S2). These times are far below the coherence time of the single atomic qubit state of $\tau_c = 75 \mu\text{s}$ (17) and the coherence time of the entangled atom-atom state,

which we expect to be at least $\tau_c/2$, and thus does not limit the quality of our experiment (agreeably, the atom-atom entanglement rate and τ_c need substantial improvement for future quantum repeater scenarios).

To evaluate the atom-atom entanglement, we perform measurements of the atomic spins in two bases. We have chosen analysis angles $\alpha = 90^\circ$ and $\alpha = 135^\circ$, while β is varied in steps of 22.5° between 90° and 180° , or between 45° and 135° , respectively. The obtained correlations are shown for the detection of the photonic $|\Psi^- \rangle_{\text{ph}}$ state (Fig. 3, A and B) and for the $|\Psi^+ \rangle_{\text{ph}}$ state (Fig. 3, C and D). By fitting sinusoidal functions to the data points, we obtain visibilities $V^{(\omega)}$ of $V_{\Psi^-}^{(90^\circ)} = 0.788 \pm 0.031$, $V_{\Psi^-}^{(135^\circ)} = 0.728 \pm 0.032$ for the $|\Psi^- \rangle_{\text{AA}}$ state and $V_{\Psi^+}^{(90^\circ)} = 0.813 \pm 0.030$, $V_{\Psi^+}^{(135^\circ)} = 0.723 \pm 0.034$ for the $|\Psi^+ \rangle_{\text{AA}}$ state, respectively. For estimation of the fidelity, we assume that the visibility in the third (unmeasured) conjugate basis is equal to the lower of the two measured ones, arriving at $F_{\Psi^-} = 0.811 \pm 0.028$ and $F_{\Psi^+} = 0.815 \pm 0.028$. These numbers prove that in both cases, an entangled state of the two atoms is generated. Moreover, the average visibilities $\bar{V}_{\Psi^\pm} = \frac{1}{2}(V_{\Psi^\pm}^{(90^\circ)} + V_{\Psi^\pm}^{(135^\circ)})$ of $\bar{V}_{\Psi^-} = 0.768 \pm 0.023$ and $\bar{V}_{\Psi^+} = 0.758 \pm 0.023$, respectively, are well above the threshold of 0.707 necessary to violate Bell's inequality.

One of our main goals is to enable a future loophole-free test of Bell's inequality (3). Inserting the data from the above measurements into $\langle \sigma_\alpha \sigma_\beta \rangle = \frac{1}{N}(N_{\uparrow\uparrow}^{(\alpha,\beta)} + N_{\downarrow\downarrow}^{(\alpha,\beta)} - N_{\uparrow\downarrow}^{(\alpha,\beta)} - N_{\downarrow\uparrow}^{(\alpha,\beta)})$,

we evaluated the parameter $S = |\langle \sigma_\alpha \sigma_\beta \rangle + \langle \sigma_\alpha \sigma_{\beta'} \rangle| + |\langle \sigma_\alpha \sigma_\beta \rangle - \langle \sigma_\alpha \sigma_{\beta'} \rangle|$ from the Clauser-Horne-Shimony-Holt-inequality $S \leq 2$, which holds for local-realistic theories (33). For the data from Fig. 3, using the settings $\alpha = 135^\circ$, $\beta = 67.5^\circ$; $\alpha = 135^\circ$, $\beta' = 112.5^\circ$; $\alpha' = 90^\circ$, $\beta' = 112.5^\circ$ together with $\alpha' = 90^\circ$, $\beta'' = 157.5^\circ$ (replacing $\beta = 67.5^\circ$), for all four heralding signals we obtain an S value exceeding the limit of 2. Because a measurement result is obtained for each and every heralding signal, the average value of $S = 2.19 \pm 0.09$ for the first time yields definite violation without relying on the fair sampling assumption for a macroscopic distance.

In this experiment, we have demonstrated heralded entanglement between two atoms 20 m apart. It was high enough to violate a Bell inequality, showing its suitability for quantum information applications such as device-independent quantum cryptography (34). The design of trap 2 allows rather straightforward extension of the distance between the two traps to at least several hundred meters, limited only by transmission of photons in the optical fiber connection. Two distant entangled atoms form the elementary link of the quantum repeater, enabling efficient long-distance quantum communication. Together with efficient and fast atomic state detection (18), this experiment forms the basis for the first loophole-free Bell experiment, answering the long-standing question on whether a local realistic extension of quantum mechanics can be a valid description of nature.

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Supplementary Materials

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Materials and Methods
Figs. S1 and S2
Table S1
Reference (35)

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Cavity Cooling Below the Recoil Limit

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Conventional laser cooling relies on repeated electronic excitations by near-resonant light, which constrains its area of application to a selected number of atomic species prepared at moderate particle densities. Optical cavities with sufficiently large Purcell factors allow for laser cooling schemes, avoiding these limitations. Here, we report on an atom-cavity system, combining a Purcell factor above 40 with a cavity bandwidth below the recoil frequency associated with the kinetic energy transfer in a single photon scattering event. This lets us access a yet-unexplored regime of atom-cavity interactions, in which the atomic motion can be manipulated by targeted dissipation with sub-recoil resolution. We demonstrate cavity-induced heating of a Bose-Einstein condensate and subsequent cooling at particle densities and temperatures incompatible with conventional laser cooling.

The discovery of laser cooling has paved the way for fundamental progress in the fields of precision spectroscopy, time and frequency metrology, quantum optics, and quantum gas physics (1–5). The essential role of repeated electronic excitation constrains the applicability of laser cooling to a limited number of atomic systems, which provide a nearly closed excitation cycle. Reabsorbed spontaneous photons yield stringent limitations with respect to the possible particle densities. The search early on for ways around the unfavorable effects of resonant excitation has led researchers to propose the use of optical cavities, which modify the electromagnetic vacuum. Cooling schemes based on optical cavities promise to be largely unaffected by density limitations and to be applicable to any polarizable matter, including molecules (6–13). Under certain conditions, cavities may even be used to cool mesoscopic objects as nanoparticles, cantilevers, or thin membranes, which has led to the new field of cavity optomechanics (14).

Optical cavities are characterized by two key figures: the rate, η , for scattering into a cavity mode relative to all free-space modes, termed Purcell factor (15), and the intracavity field decay rate, $\kappa = 1/2\tau$, which is related to the cavity bandwidth, $1/\tau$ (the spectral width of the transmission resonances), and the photon storage time, τ . Two extreme regimes arise. If $\eta > 1$, scattering into modes not supported by the cavity is practically suppressed. If $\hbar\kappa$ (where $\hbar$ is Planck's constant) is smaller than twice the recoil energy,

$E_{\text{rec}} \equiv \frac{\hbar^2 k^2}{2m}$ (m is the atomic mass, $k \equiv 2\pi/\lambda$, and λ is the optical wavelength), each atom can backscatter only a single photon. The kinetic energy

transfer required for backscattering two photons cannot be resonantly supported by the cavity, and hence further backscattering is blocked. Cavity cooling has been experimentally pioneered in the regime $\eta > 1$, $\hbar\kappa \gg E_{\text{rec}}$ for large thermal samples by using highly degenerate confocal cavities with moderate finesse and round-trip lengths on the order of cm (16, 17) and with a single or few atoms placed in 100- μm -sized high-finesse single-mode cavities (9, 10). More recently, Bose-Einstein condensates (BECs) were prepared inside such optical cavities in order to study optomechanical interactions and superradiance properties close to zero temperature (18–21). The regime $\eta < 1$, $\hbar\kappa \approx 4E_{\text{rec}}$ has been addressed in an experiment studying collective atomic recoil lasing and collective scattering in a ring cavity (22, 23). However, the experimentally highly demanding quantum regime,

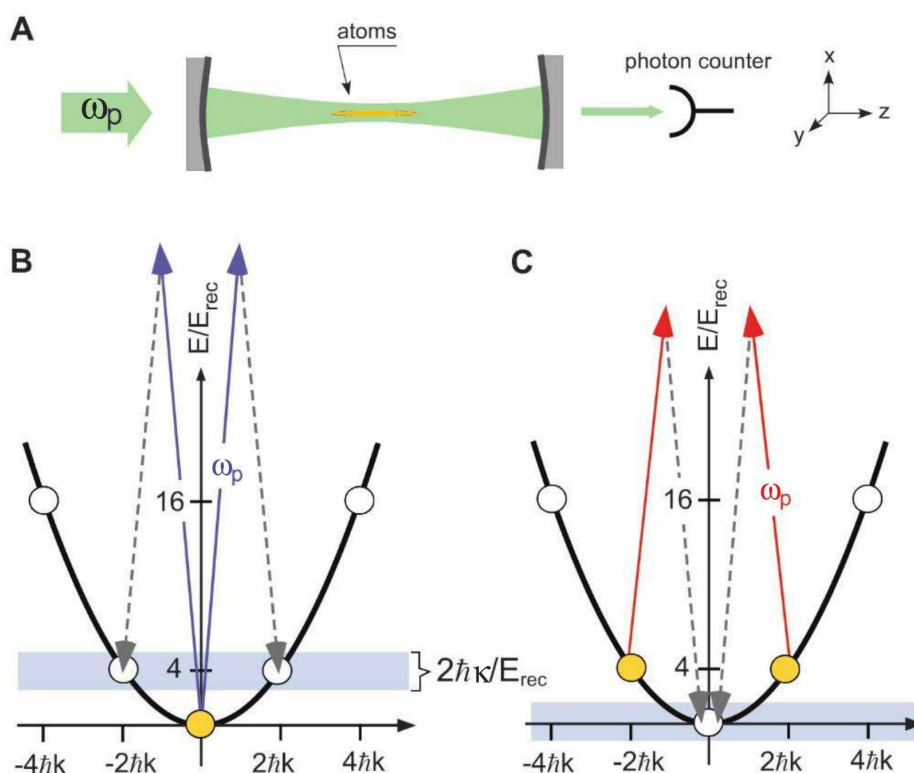


Fig. 1. Experimental scheme and basic processes. (A) Sketch of experimental set-up. (B and C) Cavity-induced heating (B) and cooling (C). The single particle dispersion is sketched by black parabolas. The possible momentum states (multiples of $2\hbar k$) connected by backscattering processes are indicated by solid disks (white if unpopulated, orange if populated). The blue (B) and red (C) solid arrows denote incident photons detuned to the blue and red sides of the cavity resonance, respectively. The dashed gray arrows represent the photons resonantly scattered into the empty cavity. The pale blue horizontal bars indicate the cavity bandwidth.

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when a Purcell factor exceeding unity ($\eta > 1$) is combined with a sub-recoil cavity bandwidth ($\hbar\kappa < 2E_{\text{rec}}$), has remained unexplored.

In our experiment, we access this regime and demonstrate targeted heating and cooling of atoms on a sub-recoil energy scale at densities on the order of 10^{14} cm^{-3} incompatible with conventional laser cooling. Our cavity-assisted cooling scheme extends the scope of laser cooling to a wide range of particles and to phase space densities close to quantum degeneracy. A weak probe beam with tunable frequency $\omega_p = c\kappa$ (c is the speed of light) is axially coupled into a linear standing wave cavity, which in the absence of atoms provides a resonance at the frequency ω_c (Fig. 1A). Below, the fundamental backscattering processes are illustrated for a single freely moving atom modeled by a quadratic dispersion relation. Figure 1B shows an atom at zero momentum irradiated with a photon at a frequency detuned by $\delta_c \equiv \omega_p - \omega_c = 4\omega_{\text{rec}}$ ($\omega_{\text{rec}} \equiv E_{\text{rec}}/\hbar$) to the blue side ($\delta_c > 0$) of the cavity resonance (blue solid arrows). The photon is scattered into the empty cavity (gray dashed arrows). A momentum of $2\hbar k$ is transferred, thus increasing the motional energy of the atom by $4E_{\text{rec}}$. Scattering of a second photon becomes impossible; this would require a second transfer of $2\hbar k$ momentum and hence an energy transfer of $16E_{\text{rec}} - 4E_{\text{rec}} = 12E_{\text{rec}}$, which cannot be provided because of the limited bandwidth of the cavity (indicated by the light blue horizontal bars). The scattered photon eventually escapes from the cavity, thus leaving the atom in either of the momentum states $\pm 2\hbar k$. Owing to the irreversibility of the momentum transfer yielding an increase of the atomic motional energy, we refer to this process as cavity-induced heating. The complementary cooling process is illustrated in Fig. 1C. The atom initially prepared in a mixture of the momentum states $\pm 2\hbar k$ is irradiated with a photon (red arrows) detuned to the red side of the cavity resonance by $\delta_c = -4\omega_{\text{rec}} < 0$. Away from the resonances $\delta_c = \pm 4\omega_{\text{rec}}$, only forward scattering is possible, yielding a shift of the cavity resonance frequency.

We prepared a BEC with up to 2×10^5 rubidium atoms (^{87}Rb) in the $F=2$, $m_F=2$ ground state close to zero temperature well confined within the mode volume of a 5-cm-long near-concentric cavity with $\kappa/2\pi = 4.5 \text{ kHz}$, $w_0 = 31.5 \mu\text{m}$ mode radius, and a finesse of $\mathcal{F} = 3.4 \times 10^5$ yielding $\eta = 43$. The atoms were held in a cigar-shaped magnetic trap with harmonic frequencies of 202, 215, and 25 Hz along the x , y , and z axes (Fig. 1A). Advanced laser stabilization techniques allowed us to axially couple a weak probe beam to the cavity and control its frequency detuning from the cavity resonance at the sub-kHz level (24). The probe wavelength $\lambda = 803 \text{ nm}$ (yielding $E_{\text{rec}}/\hbar = 2\pi \times 3.6 \text{ kHz}$) is far detuned to the red side of the relevant atomic transitions at 795 and 780 nm (D_1 and D_2 lines) with the common atomic decay rate $\Gamma = 2\pi \times 6 \text{ MHz}$, such that electronic excitation of the atoms is negligible. With

1.2×10^5 atoms, the cavity resonance is shifted by about $8 \kappa/2\pi$; that is, we operated well within the regime of strong cooperative coupling. We measured the light transmitted through the cavity with a photon counter (56% quantum efficiency) and the momentum spectrum of the atoms via time-of-flight spectroscopy [for details, see (25)].

In Fig. 2A, the probe frequency is tuned across the cavity resonance during 5 ms after a BEC with 1.2×10^5 atoms has been prepared. At certain probe detunings δ_c (indicated in the figure), momentum spectra of the atomic sample are recorded, which are shown below the upper (decreasing δ_c) and lower (increasing δ_c) frequency axes. The red transmission spectrum, showing the case of increasing δ_c , displays a resonance peak around $\delta_c/2\pi \approx -42 \text{ kHz}$. The shift with respect to the empty cavity resonance at $\delta_c = 0$ is negative in accordance with the expected normal dispersion. Although, at the resonance peak of the red transmission spectrum, the intracavity power becomes maximal, backscattering is suppressed, as is seen from the corresponding momentum spectrum of the atoms, which does not show any population of the $\pm 2\hbar k$ momentum components. Only when the probe detuning reaches $\delta_c/2\pi \approx -15 \text{ kHz}$ does backscattering according to Fig. 1B arise, and the condensate is efficiently transferred to the momentum states $\pm 2\hbar k$. The intracavity power at this point is reduced to 4 percent of its maximal value. Decreasing δ_c yields the

blue transmission spectrum. Here, the $\pm 2\hbar k$ momentum states are populated when $\delta_c/2\pi \approx -13 \text{ kHz}$ is reached, long before the resonance maximum at $\delta_c/2\pi \approx -45 \text{ kHz}$ is reached. The subsequent build-up of a significant intracavity standing wave, arising upon passing the resonance peak, does not further modify the momentum spectrum. On the red side of the resonance peak, the cooling process of Fig. 1C should become resonant; however, we do not observe a notable transfer of atoms back to the zero momentum state. We attribute this to the fact that the steep decrease of the resonance profile only allows the coupling of a few photons with appropriate frequency into the cavity as the scan proceeds. An additional obstacle is the depletion of the condensate because of multiple elastic two-body collisions, leading to rapidly increasing diffuse halos in the momentum spectra, after the states $\pm 2\hbar k$ are populated. By working at lower density, the condensate depletion could be significantly reduced.

The atom-cavity dynamics is modeled by numerically solving the one-dimensional Gross-Pitaevskii equation for the atoms

$$i\hbar \frac{\partial}{\partial t} \psi = \left(-\frac{\hbar^2}{2m} \frac{\partial^2}{\partial x^2} + \alpha^* a \hbar U_0 \cos^2(kx) + g_{1D} |\psi|^2 \right) \psi \quad (1)$$

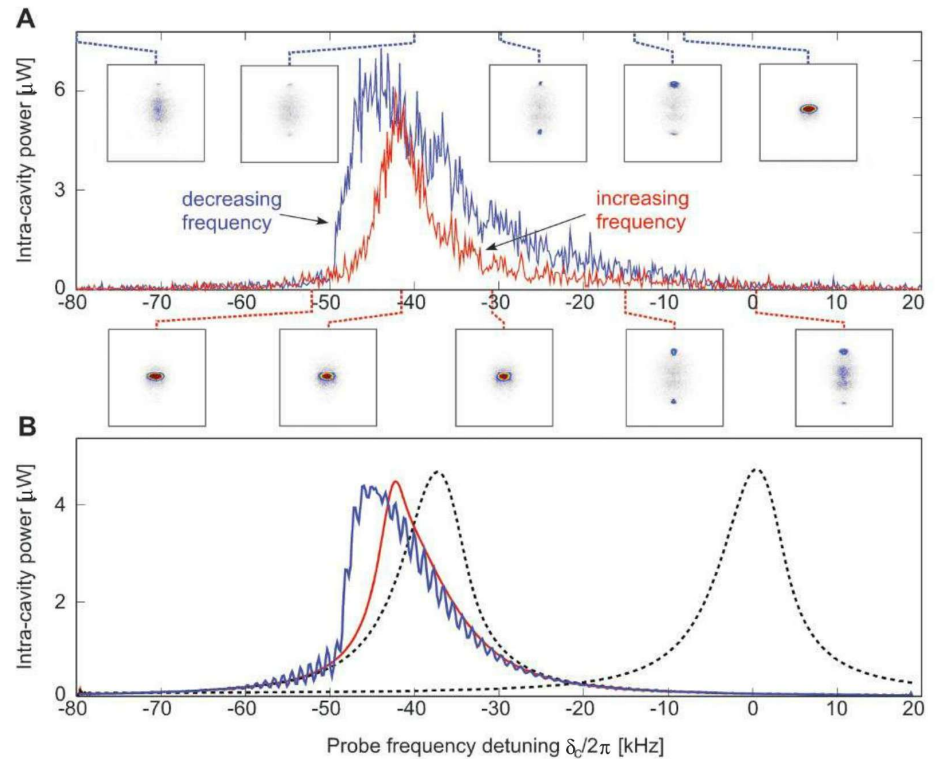


Fig. 2. Cavity transmission spectra. **(A)** Observed cavity transmission spectra for increasing (red trace) and decreasing (blue trace) probe frequencies and 5-ms scan duration. The details show corresponding momentum spectra taken at times indicated. **(B)** Model spectra. The black dashed trace peaked near -37 kHz shows the case if only forward scattering is accounted for. The black dashed trace peaked near 0 kHz shows the case of an empty cavity.

with the three-mode Ansatz $\psi(x,t) = e^{iqx}[\phi_0(t) + \phi_+(t) e^{i2kx} + \phi_-(t) e^{-i2kx}]$ and the equation

$$i\frac{\partial}{\partial t}\alpha = [-\delta_c - U_0\langle\cos^2(kx)\rangle_\psi + i\kappa]\alpha + i\zeta_p \quad (2)$$

for the intracavity field amplitude α (26). Here, $U_0 = \frac{1}{2}\eta\kappa\Gamma(S_1/\delta_1 + S_2/\delta_2)$ is the light shift per

photon, ζ_p is the rate at which the intracavity field is driven by the probe field, and g_{1D} is the effective binary collision parameter. δ_1 and δ_2 are the probe frequency detunings with respect to the relevant atomic D_1 and D_2 lines, and $S_1 = 2/3$ and $S_2 = 1/3$ are the corresponding effective line strengths for the $F=2$, $m_F=2$ ground state in the

case of σ -polarized light. The parameter q allows us to include a small initial momentum of the BEC. In contrast to previously considered scenarios, in the sub-recoil regime studied here the cavity field evolves on a time scale longer than that of the atomic dynamics, and hence Eq. 2 may not be integrated out. Furthermore, to capture the processes discussed in Fig. 1, we may not limit ourselves to contributions linear in $\phi_\pm$. In Fig. 2B, the model of Eqs. 1 and 2 is used with $q=0$ to calculate transmission spectra corresponding to the experimental observations in Fig. 2A. Although condensate depletion is neglected, the qualitative features of the observed spectra are reproduced [for details, see (25)].

In Fig. 3, we demonstrate cavity-induced heating and cooling according to Fig. 1, B and C. Initially, a BEC is prepared inside the cavity at zero momentum. A weak light pulse with 400- μ s duration and $\delta_c/2\pi = \delta_{c,\text{heat}}/2\pi \approx 0$ kHz is applied, yielding the strongly fluctuating intracavity intensity with 400-nW average power shown in the thin blue trace in the upper panel of Fig. 3A. The bottom graph shows a momentum spectrum recorded directly after the pulse terminates. The spectrum shows large populations at $\pm 2\hbar k$, whereas the initially exclusively populated zero momentum peak is practically not visible. Note that no higher momenta ($\pm 4\hbar k$) are excited, in accordance with the considerations in the context of Fig. 1. A substantial fraction of atoms (up to 75% depending on the density) forms a diffuse halo, which we attribute to multiple binary collisions. Particle loss occurs on the few percent level. In Fig. 3B, after a free evolution of the atoms with disabled probe beam [pale red vertical bar in (B)], a second pulse is applied with similar intracavity power and 200- μ s duration but with $\delta_c/2\pi = \delta_{c,\text{cool}}/2\pi \approx -43$ kHz (top graph). The subsequently recorded momentum spectrum plotted in the lower panel shows that the zero momentum peak is repopulated, whereas the momentum states $\pm 2\hbar k$, populated by the previous pulse, are completely depleted. An atom count shows that the zero momentum population after cooling exceeds the sum of the populations in all Bragg peaks ($0, \pm 2\hbar k$) previous to the second pulse by about 35%. Hence, not only the $\pm 2\hbar k$ atoms are recollected at zero momentum but a notable fraction of the halo atoms as well. For optimized pulse powers, the build-up of momentum transfer in Fig. 3, A and B, arises on a time scale of a few hundred μ s. For longer pulses, no reverse transfer is observed, in accordance with the dissipative nature of the transfer process. The fastest and most complete momentum transfer is observed for $\delta_{c,\text{heat}} - \delta_{c,\text{cool}} = 2\pi \times 43$ kHz, exceeding the condition $\delta_{c,\text{heat}} - \delta_{c,\text{cool}} = 8\omega_{\text{rec}} = 2\pi \times 28.5$ kHz naively expected according to Fig. 1. The discrepancy can be accounted for by the more realistic model of Eqs. 1 and 2 including nonlinear optomechanical dynamics (25).

We may confirm the physical picture of Fig. 1B by preparing the intracavity BEC with a small velocity $\hbar q/m$ with $q \ll k$ such that it oscillates in the magnetic trap parallel to the cavity axis with

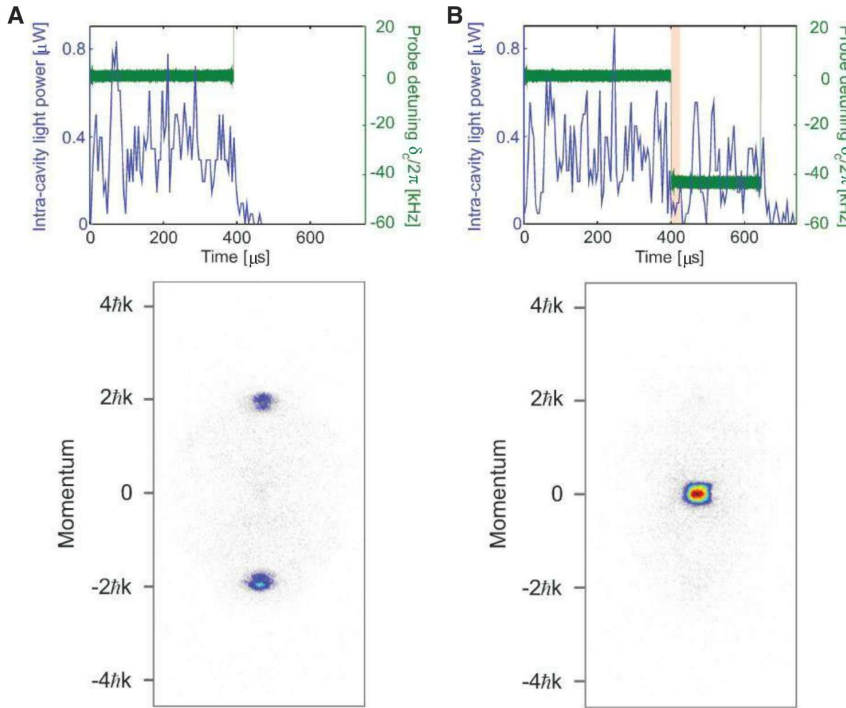


Fig. 3. Heating and cooling. (A) A 400- μ s-long pulse with $\delta_c/2\pi \approx 0$ kHz is applied to the intracavity BEC (10^5 atoms) prepared at zero momentum (top). The fluctuating blue trace shows the intracavity power. The thick green bar indicates the value of $\delta_c/2\pi$. The bottom plot shows a momentum spectrum taken directly after the pulse terminates. (B) Subsequent to the pulse applied in (A) and a 40- μ s-long free evolution with disabled probe beam [pale red vertical bar in (B)], a second pulse with 200- μ s duration and $\delta_c/2\pi \approx -45$ kHz is applied (top) with the successively recorded momentum spectrum shown in the bottom plot.

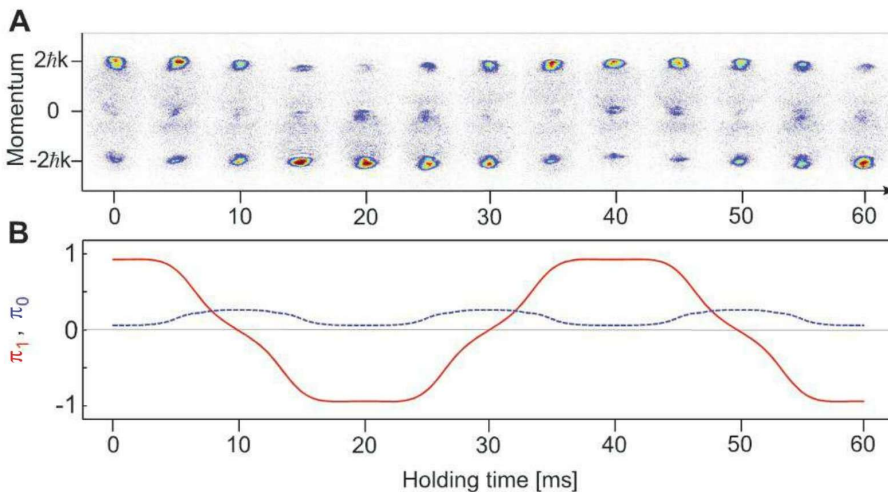


Fig. 4. Population oscillations. (A) Momentum spectra are recorded after variable holding times in the magnetic trap followed by an excitation pulse according to Fig. 3A. (B) Population ratios π_0 (blue dashed trace) and π_1 (red solid trace) calculated for the pulse applied in (A).

the harmonic frequency 25 Hz. Hence, variable holding times in the trap translate into variable initial velocities during application of the subsequent heating pulse, provided that the pulse duration (400 μ s) is much shorter than the oscillation period of about 40 ms. Because the initial velocity Doppler-shifts the backscattering resonances in Fig. 1, B and C, the populations P_n of the momentum components $n2\hbar k$, $n \in \{0, -1, 1\}$ are expected to vary periodically with the holding time. This is illustrated in Fig. 4A, where momentum spectra are shown that were recorded after application of an excitation pulse similar as in Fig. 3A following a variable holding time in the magnetic trap. The lines in Fig. 4B show the quantities $\pi_0 = P_0/(P_0 + P_{-1} + P_1)$ (blue) and $\pi_1 = (P_1 - P_{-1})/(P_0 + P_{-1} + P_1)$ (red), which have been calculated by using Eqs. 1 and 2 with $q(t) = q_0 \cos(\Omega t)$ and $q_0 \approx 0.1k$ determined by a time-of-flight measurement. The fine details of these traces largely depend on the exact values of the pulse durations, detunings, and powers. Nevertheless, the coarse structure describes well the observations in Fig. 4A (25).

Cavity-induced sub-recoil momentum transfer could be used to also cool hotter, truly thermal samples by applying sequences of pulses with appropriately adjusted frequency detunings. Cross-dimensional relaxation because of ergodic mix-

ing in the external potential and elastic collisions should allow us to cool all three dimensions. The achievement of quantum degeneracy starting from a thermal sample well above the critical temperature is an intriguing goal, which appears to be in reach with our cooling scenario.

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Supplementary Materials

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Large Volcanic Aerosol Load in the Stratosphere Linked to Asian Monsoon Transport

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The Nabro stratovolcano in Eritrea, northeastern Africa, erupted on 13 June 2011, injecting approximately 1.3 teragrams of sulfur dioxide (SO₂) to altitudes of 9 to 14 kilometers in the upper troposphere, which resulted in a large aerosol enhancement in the stratosphere. The SO₂ was lofted into the lower stratosphere by deep convection and the circulation associated with the Asian summer monsoon while gradually converting to sulfate aerosol. This demonstrates that to affect climate, volcanic eruptions need not be strong enough to inject sulfur directly to the stratosphere.

Aerosols in the stratosphere have an important influence on climate variability: They scatter incident sunlight, thus cooling Earth's surface, but with a highly variable effect owing to the size and frequency of volcanic

eruptions. Sustained aerosol concentrations in the stratosphere are supported by particles with lifetimes of a year or longer, mainly sub-micrometer hydrated sulfuric acid droplets rather than solid particles, such as volcanic ash, which due to their size settle from the stratosphere more quickly. Sulfuric acid droplets form at stratospheric altitudes through oxidation of gases containing sulfur, mainly carbonyl sulfide and sulfur dioxide (SO₂) (1). For a volcanic eruption to affect stratospheric aerosol substantially, it typically has to be sulfur-rich and energetic enough to penetrate the tropopause. The Nabro stratovolcano in Eritrea, northeastern Africa, erupted

on 13 June 2011, with ash clouds reaching altitudes of 9.1 to 13.7 km (2). Nadir measurements made by the Ozone Monitoring Instrument (OMI) show that the total emitted SO₂ was approximately 1.3 Tg (3). These reported eruption heights are below tropical tropopause levels, which are 16 km or higher. In spite of this, scattered sunlight measurements from the limb-scanning Optical Spectrograph and Infra-Red Imaging System (OSIRIS) satellite instrument (4) show that Nabro caused the largest stratospheric aerosol load in the decade-long OSIRIS measurement record. Additionally, the OSIRIS measurements show the aerosol enhancement building most strongly above the Asian monsoon, which has recently been identified as a potentially effective pathway for transport of air from the troposphere to the stratosphere (5) and a potentially important region for the formation of small stratospheric aerosol particles during volcanically unperturbed conditions (6, 7).

Global measurements of the stratospheric aerosol load have been made by limb-scanning solar occultation satellite instruments for decades. These aerosol extinction measurements, in particular from the Stratospheric Aerosol and Gas Experiment (SAGE) series of instruments (SAGE, 1979 to 1981; SAGE II, 1984 to 2005; and SAGE III, 2002 to 2005), have provided an essential understanding of the stratospheric aerosol layer. SAGE II measurements were especially important for tracking and quantifying the eruptions of El Chichón in 1982 (8) and Mount Pinatubo in 1991 (9, 10), both of which caused

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considerable cooling of the lower troposphere in the 2 years after the eruption (11, 12).

Since the eruption of Mount Pinatubo in 1991, there have been no major volcanic eruptions that have substantially increased the stratospheric aerosol load. However, in the past decade an increase in the so-called “background” level has been observed. Ground-based lidar measurements show that the stratospheric aerosol load has increased since 2000 at 5 to 7% per year (6). Suggested causes of this increase range from increased anthropogenic emissions (6) to variability associated with a series of small, typically

tropical, volcanic eruptions (13). This “persistently variable” increase in the background level has been shown to cause an additional radiative forcing of -0.1 W/m^2 over the past decade, effectively reducing the impact of greenhouse gas increases over the same time period (14). The effects of these relatively minor eruptions are important to include for climate model predictions in order to avoid overestimation of radiative forcing and thus global warming. The effect of the 2011 Nabro eruption will extend the negative forcing from the stratospheric aerosol changes observed over the past decade.

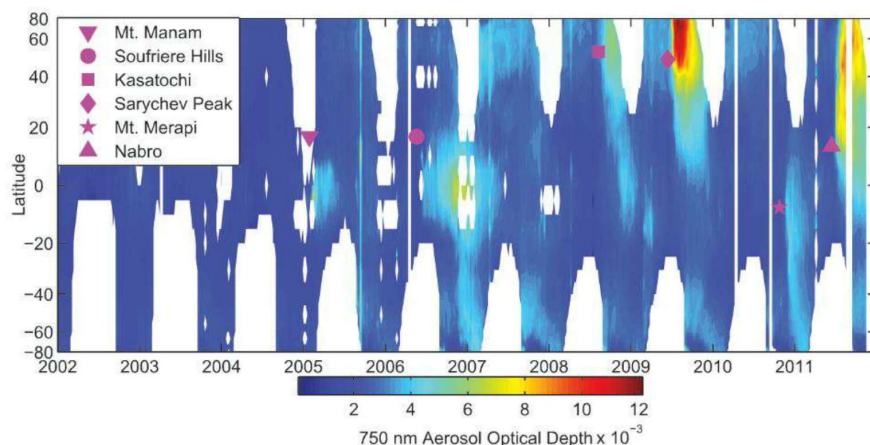


Fig. 1. The weekly zonal mean stratospheric aerosol optical depth at 750 nm for all measurements during the OSIRIS mission. The locations and times of the six volcanic eruptions with the largest stratospheric effect in the measurement record are noted with the markers. The yearly time label indicates 1 January of each year.

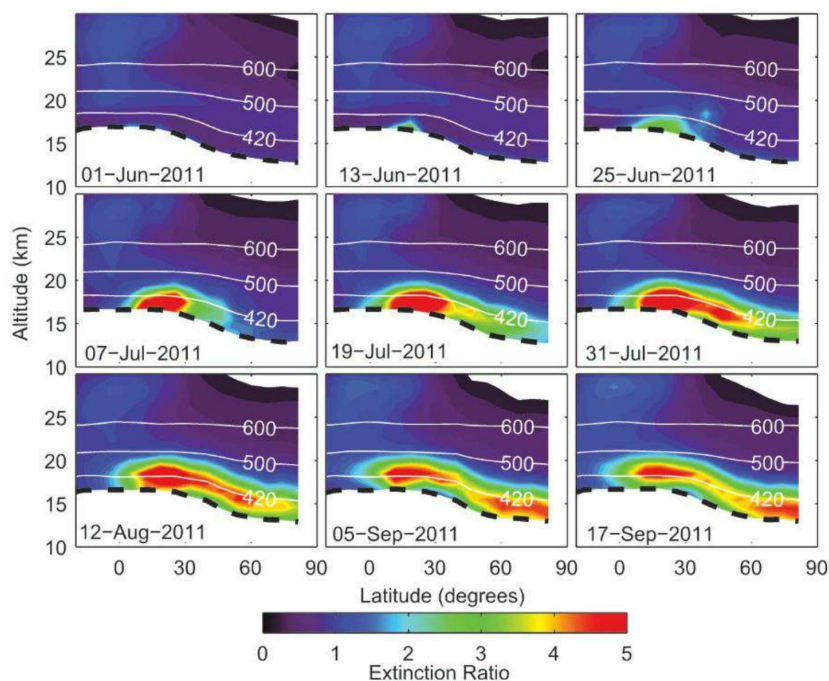


Fig. 2. Altitude profiles of the zonal average aerosol extinction ratio, over all longitudes, for 12-day time periods from June through September 2011, showing the progression of the stratospheric aerosol enhancement from the Nabro eruption on 13 June. The altitude of the 380 K level of potential temperature is shown by the dashed line with white contours (kelvin) for higher isentropic levels.

The OSIRIS instrument on the Odin spacecraft (4, 15), launched in 2001, measures the spectrum of limb-scattered sunlight, providing vertical aerosol profiles and almost daily coverage of the sunlit globe. The measurements are used with a radiative transfer code to retrieve vertical profiles of stratospheric aerosol extinction coefficient at 750 nm (16, 17). These agree with coincident SAGE III occultation measurements to within 10% during the 4 years of mission overlap, 2001 to 2005 (18). The time series of OSIRIS measurements of stratospheric aerosol optical depth over the past 10 years (Fig. 1) shows the large effect the recent Nabro eruption, 13 June 2011, had on the entire Northern Hemisphere, with zonal mean optical depths reaching some of the largest values in the entire OSIRIS record. The stratospheric aerosol optical depth is calculated for altitudes above 380 K potential temperature. The 380 K level corresponds to the altitude of the thermal tropopause in the tropics—typically 16 to 17 km—and is approximately the middle of the so-called tropical tropopause layer, or TTL (19). The use of this lower bound ensures that the optical depth shown is not due to tropospheric aerosol entering the lowermost stratosphere in the mid- and high latitudes, where air below the 380 K level and above the thermal tropopause can enter the stratosphere through quasi-horizontal isentropic transport. Optical depths above the background typical of the early OSIRIS years are also evident after earlier tropical volcanic eruptions, notably Mount Manam, January 2005; Soufrière Hills, May 2006; and Mount Merapi, October 2010. The northern high-latitude eruptions of Mount Kasatochi, August 2008, and Sarychev Peak, July 2009, both caused a notable effect on the stratospheric optical depth that extended to lower latitudes in the months after the eruption (20–22).

Tropical eruptions are particularly important to consider when analyzing global climate because stratospheric aerosol has a long lifetime in the tropical stratosphere and can be transported into the mid-latitudes of both hemispheres, as was the case with the Mount Manam eruption in 2005 and the Soufrière Hills eruption in 2006. In contrast, the volcanic aerosol enhancement from the Nabro eruption was largely confined to the Northern Hemisphere. The aerosol plume reached altitudes up to 21 km (Fig. 2), with rapid transport to higher latitudes following sloping isentropic levels. The maximum of the enhanced aerosol extinction ratio occurred at approximately 30°N latitude, substantially offset from the Nabro volcano at 13°N. There is a clear difference in the distribution of aerosol from Soufrière Hills and Nabro eruptions, which occurred within 3° of latitude and within 1 month in time of year of each other. The Soufrière Hills eruption reached altitudes of up to 20 km (23), which is well above the TTL, and the resulting aerosol circulated within the so-called tropical pipe, where mixing to mid-altitudes is weak (24, 25). The Nabro eruption however, reached to much lower height and only attained stratospheric altitudes

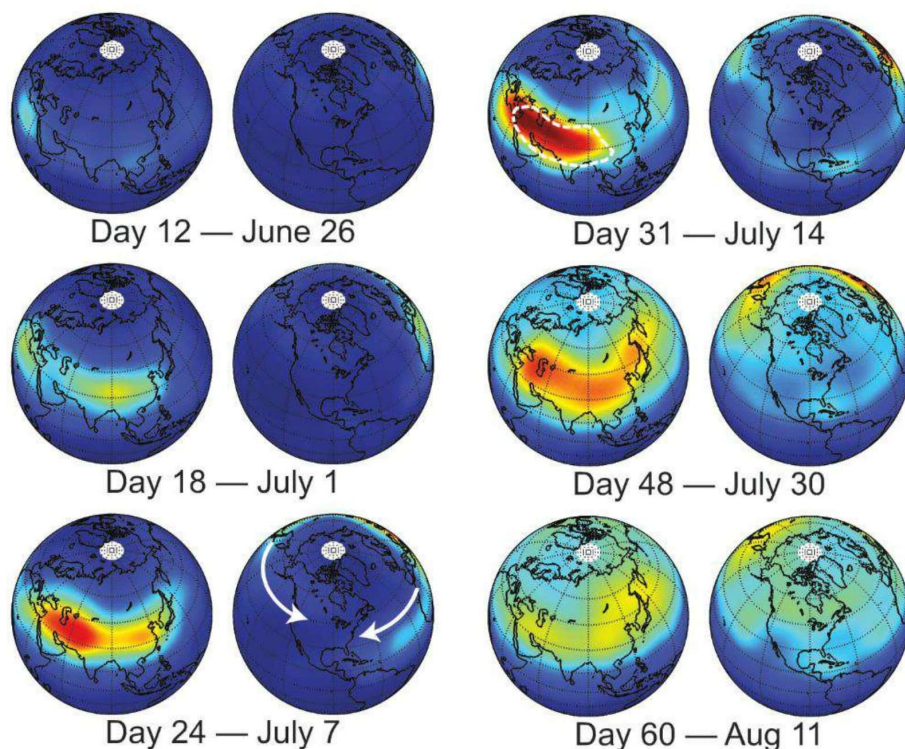


Fig. 3. Maps of the mean stratospheric aerosol optical depth at 750 nm for a series of selected days after the Nabro eruption. Each set of two maps, which are centered on Asia and North America, is a 5-day average, in which the day indicated is day 3 of the 5 days used in the average. White arrows indicate the direction of the plume transport. The dashed white line on the map for 14 July is a specific contour of geopotential height for 12 to 16 July that indicates the region of the Asian monsoon anticyclone (fig. S1).

through subsequent transport processes associated with deep convection (fig. S3).

The history of stratospheric aerosol optical depth over the Northern Hemisphere (Fig. 3) shows that the aerosol enhancement built while remaining confined for several weeks to the region between central Asia and the Middle East. The zonal and meridional winds and geopotential height from the National Centers for Environmental Prediction (NCEP) at 200 and 100 hPa—approximately 13.5 and 16.1 km, respectively (fig. S1)—show the strong Asian monsoon anticyclone, which existed from June through September over Asia and the Middle East, where the volcanic aerosol was observed. The monsoon anticyclonic vortex is known to provide an effective but slow vertical transport of tropospheric air to the stratosphere and confinement of the air in the lower stratosphere (26). For example, enhanced stratospheric hydrogen cyanide (HCN), measured by the Atmospheric Chemistry Experiment (ACE) satellite, are well correlated with the Asian monsoon region (5), and Cloud-Aerosol Lidar and Infrared Pathfinder Satellite Observations (CALIPSO) measurements have identified a coincident layer of enhanced aerosol concentrations (7). Vertical transport to stratospheric altitudes is particularly effective on the eastern side of the monsoon circulation (26), which is where a large stratospheric optical depth

enhancement was first observed on 1 July, 18 days after the eruption (Fig. 3). At the same time, smaller but still enhanced optical depths were observed over North Africa, much closer to the Nabro eruption. CALIPSO measurements on 16 June show a large aerosol layer over North Africa at 10 to 13 km altitude colocated with a Global Ozone Monitoring Experiment-2 (GOME-2) observation of the SO₂ plume (fig. S2) and largely in good agreement with the reported eruption altitude. The NCEP winds at this level (fig. S1) are also consistent with the SO₂ plume dispersion; the plume is transported in the anticyclonic monsoon circulation from Africa to eastern Asia over a few days. CALIPSO measurements over both North Africa and the monsoon region on 1 July show extended aerosol layers at altitudes up to 20 km (fig. S3); the depolarization signature of these layers indicates spherical, presumably hydrated sulfate, aerosols originating from the volcanic SO₂. Together, these satellite measurements indicate rapid vertical transport of SO₂ through the TTL to the lower stratosphere. The deep convective activity associated with these geographic regions, which penetrates to approximately 17 km according to CALIPSO measurements (fig. S3), demonstrates that the rapid vertical transport of air by convection—largely thought to play a dominant role only below the TTL—can provide a rapid pathway to the stratosphere.

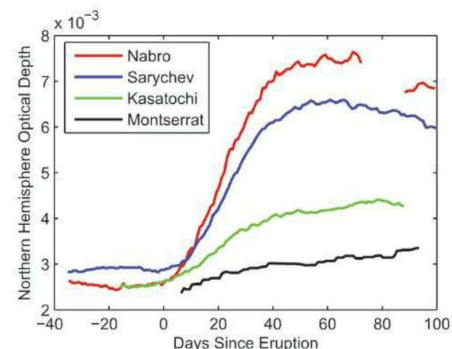


Fig. 4. The time series of Northern Hemisphere stratospheric aerosol optical depth during days after the volcanic eruptions that caused the four largest stratospheric perturbations as measured by OSIRIS since its launch in 2001. Soufrière Hills Montserrat (16.72°N, 62.18°W) erupted on 20 May 2006, Kasatochi (52.1°N, 175.3°W) erupted on 8 August 2008, and Sarychev (48.1°N, 153.2°E) began erupting on 12 June 2009. The reported SO₂ emissions of these eruptions were 1.3 Tg from Nabro (3), 1.2 Tg from Sarychev (22), 1.5 Tg from Kasatochi (20), and 0.1 Tg from Soufrière Hills Montserrat (23).

In later July, large values of optical depth were observed constrained to the region over Asia and the Middle East, until early August, when the enhanced aerosol dispersed and quickly circulated throughout the Northern Hemisphere, likely because of weakening of the anticyclone near the end of the monsoon. An interesting feature of the aerosol circulation was a small plume of westward transport at latitudes close to the equator and a second higher-latitude small plume undergoing eastward transport. These two plumes merged along the western side of North America on 14 July, 31 days after the eruption. In situ balloon measurements of stratospheric aerosol above Laramie, Wyoming (27), confirm the transport of volcanic aerosol over North America and provide estimates of particle size and concentration (fig. S4) at 46 and 140 days after the eruption.

A comparison of the evolution of daily Northern Hemisphere optical depth for the four largest volcanic eruptions in the OSIRIS record is shown in Fig. 4. The Northern Hemisphere optical depth is found by integrating the point measurements of optical depth for each day in a 2-week window over the hemispheric area. Peaking around 60 days after the eruption, the Nabro eruption is responsible for the largest stratospheric aerosol optical depth in the record and is approximately 15% larger than Sarychev, which was previously the largest. Given that the stratospheric aerosol burden was extremely low in the late 1990s and early 2000s because of the lack of any major volcanic eruptions, the Nabro eruption has caused the largest stratospheric aerosol burden since Mount Pinatubo. The impact of Nabro, a relatively low-altitude eruption, appears

to have been enhanced by its timing and location, allowing the Asian monsoon anticyclone to enhance the vertical transport while confining the majority of the aerosol to Asia and the Middle East until August, rather than rapidly mixing zonally and spreading throughout both hemispheres. The negative radiative forcing resulting from the 2011 Nabro eruption continues the trend from small eruptions of the past decade (13, 14), but the inherently variable nature of volcanic eruptions means that any short-term future cooling of the surface from volcanic stratospheric aerosol is uncertain.

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Supplementary Materials

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Figs. S1 to S4

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ENSO Drove 2500-Year Collapse of Eastern Pacific Coral Reefs

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Cores of coral reef frameworks along an upwelling gradient in Panamá show that reef ecosystems in the tropical eastern Pacific collapsed for 2500 years, representing as much as 40% of their history, beginning about 4000 years ago. The principal cause of this millennial-scale hiatus in reef growth was increased variability of the El Niño–Southern Oscillation (ENSO) and its coupling with the Intertropical Convergence Zone. The hiatus was a Pacific-wide phenomenon with an underlying climatology similar to probable scenarios for the next century. Global climate change is probably driving eastern Pacific reefs toward another regional collapse.

Global climate change is altering coral reef ecosystems through increasing sea temperatures and declining carbonate saturation states (1, 2). Warmer and more acidic conditions inhibit coral calcification, carbonate precipitation, and submarine cementation (3–5). These effects are expected to reduce long-term rates of reef framework construction. Here we provide an explicit test of this prediction by showing how vertical reef accretion in the tropical eastern Pacific (TEP) responded to climatic oscillations during the Holocene. Increased variability of the El Niño–Southern Oscillation (ENSO) ~4000 years ago produced conditions in the TEP similar to those expected under plausible scenarios of future climate, stalling reef

accretion off the Pacific coast of Panamá for 2500 years.

Living coral assemblages in the TEP respond to two principal environmental drivers: upwelling on a seasonal scale (6) and ENSO on a multiannual scale (7). The depressed water temperatures and reduced pH levels that accompany seasonal upwelling reduce coral growth (6). High sea temperatures associated with El Niño events cause bleaching, which reduces coral growth or kills corals outright (7). Mass coral mortality has been followed by intense bioerosion and the net loss of reef framework on a multidecadal scale (8). Upwelling and El Niño events are thought to account for generally poor Holocene reef development in the TEP (6, 7, 9), but neither has

been explicitly linked to millennial-scale rates of reef accretion. We investigated the history of reef framework construction along an upwelling gradient in Pacific Panamá and evaluated the influences of seasonal upwelling and ENSO on the tempo and mode of reef development.

The uncemented reef frameworks of the TEP consist of coral fragments packed in fine sediment. We extracted 14 push-cores from subtidal reef-slope habitats on three reefs across Pacific Panamá with distinct upwelling regimes (10). Isla Contadora, in the Gulf of Panamá, experiences intense seasonal upwelling; upwelling is intermediate at Isla Iguana, also in the Gulf of Panamá; and there is no upwelling at Isla Canales de Tierra in the Gulf of Chiriquí

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(Fig. 1). This upwelling gradient was established at least as early as the mid-Holocene (11) (table S1).

Each core was extruded and sectioned into 5-cm intervals. The coral constituents of the intervals were sorted by species and taphonomic condition to identify layers representing different modes of reef development. Layers were dated with three methods: ^{14}C analysis by standard techniques and accelerator mass spectrometry (AMS), and U/Th analysis by inductively coupled plasma mass spectrometry (ICP-MS). Radiocarbon dates were calibrated using the marine calibration curve and the local reservoir correction (fig. S1 and tables S1 and S2). Accretion rates were calculated by dividing the depth range of each interval in the framework by its time span (10).

The cores were dominated by well-preserved skeletons of *Pocillopora damicornis* (Fig. 2A), which is the primary constructor of modern reef framework in the TEP. A sample of well-preserved *Pocillopora*, resting directly atop the basaltic bedrock in one core, indicated that reef growth had begun by 6900 calibrated calendar

years before the present (cal yr B.P.) (where the present is 1950). Each core contained a narrow interval dominated by a combination of taphonomically degraded *Pocillopora* rubble, branching coralline algae, and *Psammocora stellata*, which does not build framework. This narrow interval represents a millennial-scale hiatus in active reef development, from 4220 to 4064, until 1820 to 1520 cal yr B.P.

Despite concentrated dating of corals within and around the interval of limited deposition in the cores (25 standard, 16 AMS, and 19 ICP-MS dates), no samples of *Pocillopora* dated within the interval 4064 to 1820 cal yr B.P. Dated samples of *Psammocora* in the hiatus (12 AMS and 5 ICP-MS dates) indicated that this coral stopped growing by 4332 cal yr B.P. (2σ range: 4442 to 4186). *Psammocora* recovered by 2384 cal yr B.P. at the earliest (2σ range: 2522 to 2289); thus, coral growth restarted several centuries before framework accretion resumed.

Accretion rates were significantly reduced at all sites during the hiatus as compared with intervals of active reef growth before and after [Fig. 2B and table S3; analysis of variance

(ANOVA), $F_{2,29} = 64.001$, $P < 0.001$, Tukey's honestly significant difference (HSD) $P < 0.001$]. During active reef growth, average rates of vertical accretion were similar among our sites and were comparable to rates for Caribbean reefs (table S4). The slower overall accretion rates in the Gulf of Panamá reported previously (9) resulted from differences in the timing of the hiatus among environments. The hiatus began at approximately the same time at all sites (ANOVA $F_{2,9} = 2.276$, $P = 0.158$; table S5); however, it ended later and lasted longer at Contadora, the strong-upwelling site, as compared with Canales de Tierra, where there was no upwelling (ANOVA, $F_{2,9} = 8.390$, $P = 0.009$; $F_{2,9} = 6.189$, $P = 0.020$; Tukey HSD, $P < 0.05$, tables S6 and S7). No significant differences were found in the timing of the hiatus between either site and Iguana (Tukey HSD, $P > 0.05$).

Contemporaneous millennial-scale hiatuses in reef development have been recorded in Golfo Dulce, Costa Rica (12), and in inshore sections of the Great Barrier Reef and Moreton Bay, Australia (table S8) (13, 14). Likewise, an intermittent hiatus in Japan coincided with the onset of the hiatus in Pacific Panamá (15). These events, previously attributed to local- or regional-scale phenomena, are more likely the result of Pacific-wide climatic changes that disrupted reef development in the nearshore environments of many regions simultaneously.

The hiatus corresponds to a time of enhanced climatic variability (Fig. 3). ENSO frequency and intensity increased beginning 4500 to 4000 cal yr B.P. (16–20), coincident with the onset of the hiatus in our cores. A concurrent period of high variability in the latitudinal migration of the Intertropical Convergence Zone (ITCZ) (Fig. 3B) suggests that the coupled influence of ENSO and the ITCZ caused the increase in ENSO strength (16, 19); reconstructions suggest that El Niño events occurring 4000 to 2000 cal yr B.P. were among the strongest of the Holocene (18) (Fig. 3C).

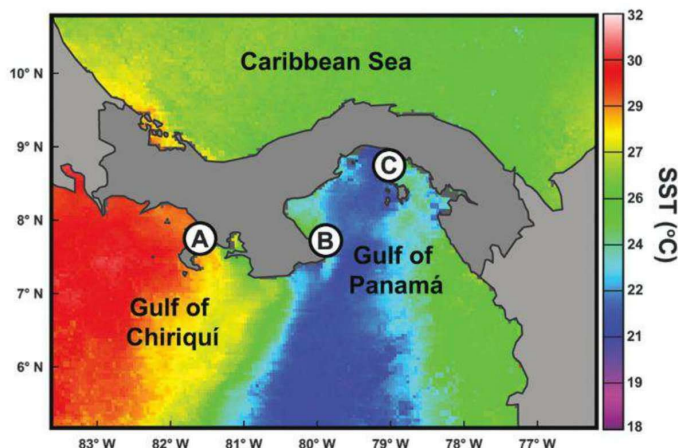
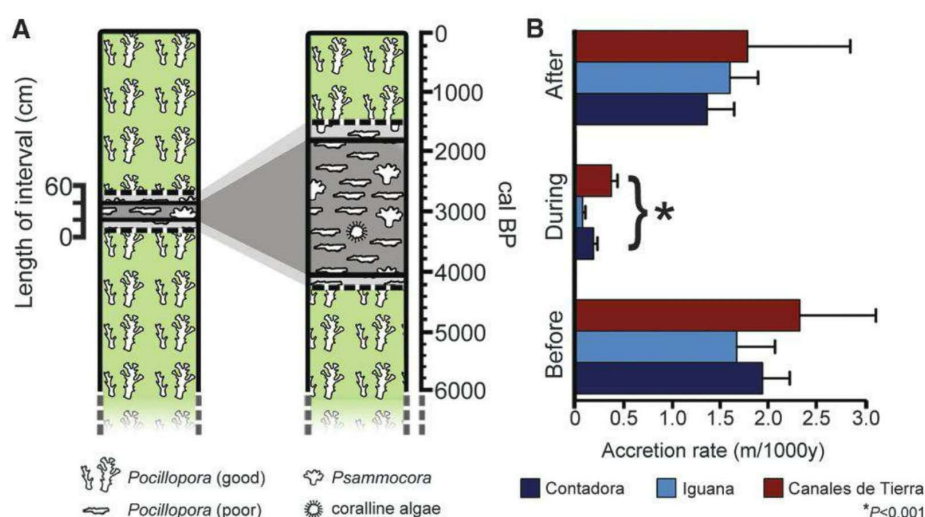


Fig. 1. Map of Pacific Panamá showing the locations of study reefs in relation to upwelling regimes. A, Canales de Tierra. B, Iguana. C, Contadora. The coloration shows sea surface temperature (SST) at the peak of the 2009 upwelling season, 4 to 17 March. The image was created from MODIS/Aqua Satellite SST data using NASA's POET v.2.0 software (<http://poet.jpl.nasa.gov/>).

Fig. 2. (A) Composite core log. Green shading with icons of *Pocillopora* in good taphonomic condition indicates periods of active reef growth. Gray shading with icons of *Pocillopora* in poor condition, *Psammocora*, and coralline algae indicates interrupted reef accretion. The length of the hiatus (gray shading) is shown expanded to depth in the reef framework (left) and in calibrated calendar years before the present (right). Dark gray shading represents the most conservative time span for the hiatus; light gray shading shows the maximum range based on all three sites. (B) Mean accretion rates before, during, and after the hiatus. Error bars represent standard errors; the asterisk indicates significant difference from the other groups ($P < 0.001$).



Very strong El Niño events beginning ~4200 cal yr B.P. (Fig. 3D) (16–18, 20, 21) could have caused the initial collapse of coral populations in Pacific Panamá because of bleaching-related mortality (7). ENSO activity peaked ~3000 cal yr B.P. (Fig. 3E), with stronger and more frequent El Niño events than at any other time during the Holocene (20–22). Frequent high-temperature anomalies would have precluded the recovery of coral populations, suppressing reef accretion.

More frequent La Niña events 3800 to 3200 cal yr B.P. (19) would also have inhibited reef development. Increased precipitation in Panamá during La Niña events (23) would have elevated turbidity and reduced light levels (14). On the high-turbidity reefs of the TEP (12), small changes in water clarity would likely have reduced light

levels below the threshold necessary for active development (24). Greater atmospheric pressure across the Pacific under La Niña conditions would have lowered sea levels in the TEP (25), subjecting corals more frequently to lethal subaerial exposure. Frequent thermal anomalies, reduced light fields, and repeated subaerial exposure all would have limited accretion rates during the hiatus.

ENSO activity continued to increase after the end of the hiatus (17, 19, 26); however, major changes in the mode of ENSO coincided with its termination (16, 18, 19). ENSO and the ITCZ decoupled ~2500 cal yr B.P. (19), accounting for the decline in variability of the ITCZ (16). There was also a reduction in La Niña activity ~2000 to 1500 cal yr B.P. (19, 27), which would have increased regional light availability and reduced

the frequency of subaerial exposure. Although the absolute number of El Niño events may have increased beginning 2000 cal yr B.P. (17, 19, 26, 27), the events were probably stronger during the hiatus (18). The waning influence of La Niña and reduction in El Niño strength apparently permitted active accretion to resume 1820 to 1520 cal yr B.P.

We propose that shifts in the frequency and intensity of ENSO, and especially its coupling with the ITCZ, were the ultimate cause of the depositional hiatus at our sites in Pacific Panamá and, perhaps, elsewhere in the Pacific (Fig. 4). Coral populations may have persisted on nearby reefs or in favorable microhabitats, facilitating the eventual recolonization of the reef slopes at Contadora, Iguana, and Canales de Tierra. Although climate change has been linked to reef development in deep time (28), sea-level changes have heretofore been considered the major control on reef development during the Quaternary (29). Our study, in contrast, implicates climatic oscillations as the primary driver of reef development during the Holocene. Reefs of the TEP are poorly developed, but not because coral growth was suppressed during their entire history by low sea temperatures and elevated partial pressure of CO₂ from upwelling, or by high sea temperatures from El Niño events with modern characteristics (3, 7). The reefs are poorly developed because coral populations collapsed for an interval spanning ~40% of their 6000- to 7000-year existence.

Alternative hypotheses fail to explain the duration, timing, or spatial extent of the hiatus. A sea-level highstand occurred in many parts of the Pacific ~6000 cal yr B.P. (13). Reduced light levels in Pacific Panamá could have drowned reefs at that time; however, there is no evidence of such a highstand in the TEP (30), and peak sea levels elsewhere occurred two millennia before the onset of the hiatus. The opposite scenario, in which the reefs filled the available accommodation space during the (putative) highstand and then stopped growing (13), cannot explain why vertical accretion abruptly resumed at a time when eustatic sea level was either still declining from the highstand or continuing a multimillennial rise (13, 31). Our sites were located well within the subtidal envelope from 6000 cal yr B.P. to the present (30), and there is no evidence of tectonic connections that would have allowed uplift or subsidence uniformly from the Gulf of Panamá to Golfo Dulce (32). The Pacific-wide distribution of the hiatus eliminates localized fluctuations of relative sea level or tectonics as the controlling influence.

Finally, extensive bioerosion of the subfossil framework by echinoids did not create an unconformity we detected as the hiatus. Although rates of bioerosion after ENSO-related disturbance can exceed rates of carbonate production (8), bioerosion does not cause significant information loss from the fossil record once the

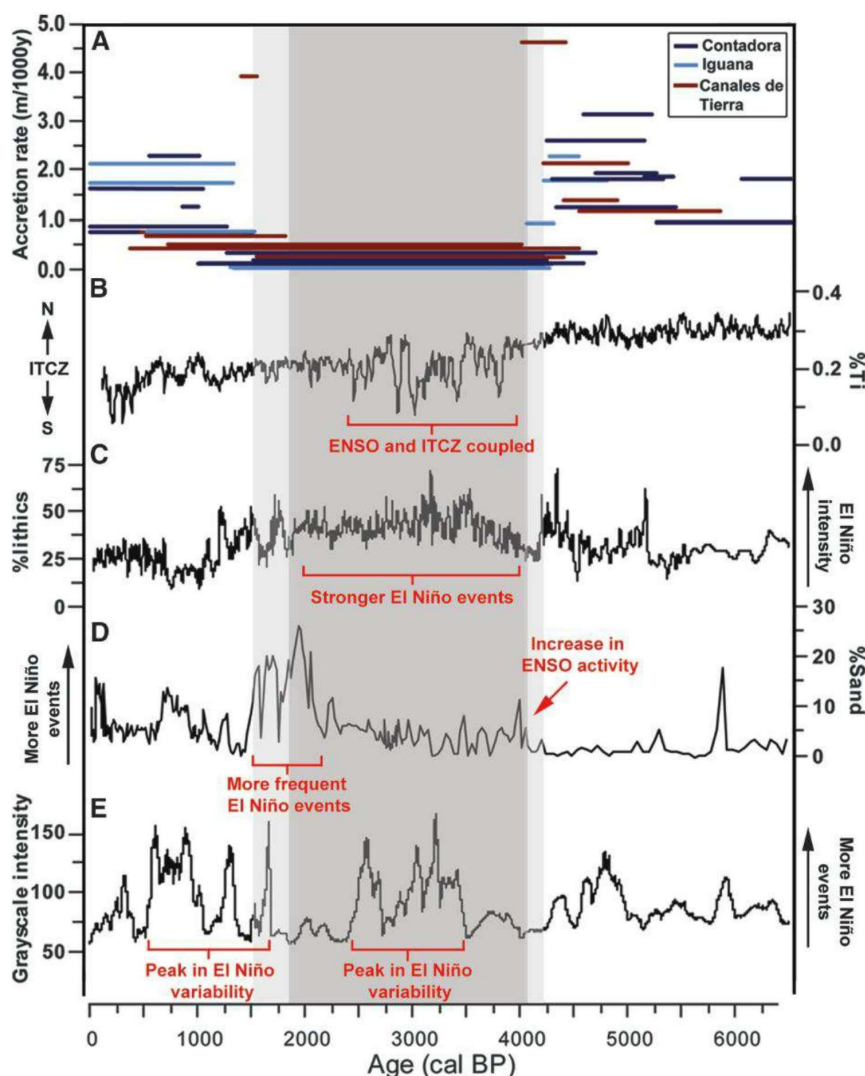


Fig. 3. Climatic reconstructions of ENSO-related precipitation as compared with accretion rates in the TEP. (A) Reef accretion, this study. (B) Percent of titanium in a deep-sea core from Cariaco Basin (16). (C) A 10-year running mean of the relative percent of lithic sediments in a deep-sea core off the coast of Peru (18). (D) Percent of sand in a core from El Junco Lake, San Cristobal, Galápagos (19). (E) A 50-year running mean of grayscale intensity in a core from Laguna Pallacocha, Ecuador (26). Gray shading is as in Fig. 2.

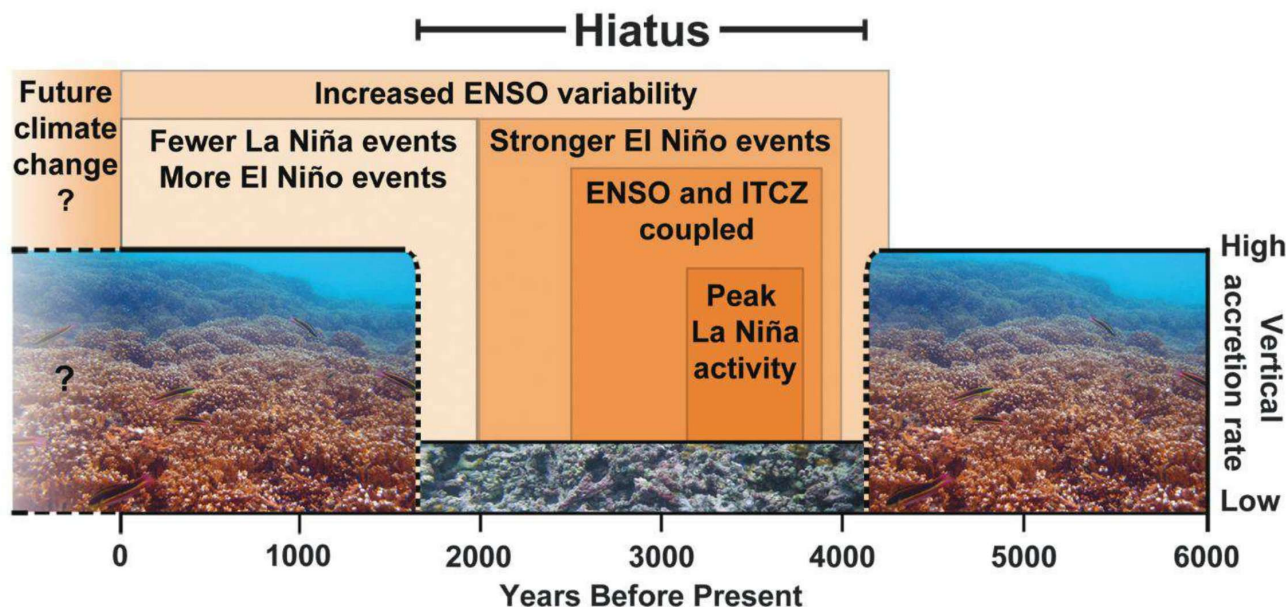


Fig. 4. Conceptual model of the climatic drivers of reef collapse in the TEP. Temporal ranges of climatic conditions are shown in orange, and community states are illustrated pictorially.

coral skeletons have been stabilized and packed in fine sediment. In our cores, the uppermost, open framework generally represented less than 100 years of depositional time. Bioerosion cannot explain the 2500-year hiatus, and the error due to bioerosion in estimating its timing is negligible. Furthermore, extensive bioerosion could be a modern phenomenon resulting from overfishing of the predators of echinoids (33).

Reef dynamics in the TEP and elsewhere in the Pacific have been driven by long-term shifts in ENSO variability for at least the past 6000 years. The intensity of seasonal upwelling acted as a second-order process, potentially influencing local ecosystem resilience. Ecological processes, including herbivory, corallivory, and competition, exerted imperceptible third-order effects on long-term rates of reef accretion, although biological interactions in refuges could have influenced the recovery of coral populations. The widespread distribution of the hiatus suggests that climatic variability was a controlling influence on reef accretion over a broad longitudinal range in the Pacific during the Holocene.

In recent years, ENSO activity has devastated tropical reefs (34). Enhanced ENSO-like conditions in a warming world (25) could once again put Pacific reefs at risk of collapse. The TEP is a low-diversity system in which a modern history of intense disturbance has driven acclimation and adaptation in the coral populations. Higher-diversity reef systems in the western Pacific that have experienced a relatively benign history of disturbance could be even more vulnerable to climate change (35). But if Pacific reefs were able to recover after a millennial-scale hiatus in coral growth, and if current trends in CO₂ emis-

sions can be stopped or reversed, reefs of the future might also prove resilient.

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Supplementary Materials

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Materials and Methods
Fig. S1
Tables S1 to S8
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The Dynamics of Cooperative Bacterial Virulence in the Field

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Laboratory experiments have shown that the fitness of microorganisms can depend on cooperation between cells. Although this insight has revolutionized our understanding of microbial life, results from artificial microcosms have not been validated in complex natural populations. We investigated the sociality of essential virulence factors (crystal toxins) in the pathogen *Bacillus thuringiensis* using diamondback moth larvae (*Plutella xylostella*) as hosts. We show that toxin production is cooperative, and in a manipulative field experiment, we observed persistent high relatedness and frequency- and density-dependent selection, which favor stable cooperation. Conditions favoring social virulence can therefore persist in the face of natural population processes, and social interactions (rapid cheat invasion) may account for the rarity of natural disease outbreaks caused by *B. thuringiensis*.

The growth and virulence of pathogenic bacteria often depends on cooperation between bacterial cells (1). Bacteria produce and release a range of extracellular factors that perform a wide range of functions, including nutrient acquisition, host-cell lysis, biofilm formation, quorum sensing, and immune evasion (2–4). The benefits derived from producing these extracellular factors are often shared with neighboring cells and so represent a form of cooperation (5) analogous to what economists would call the production of a “public good.” The problem with such cooperation is that it is susceptible to “cheating” by cells that benefit from the cooperative behavior of others, without paying the cost of cooperation themselves (2, 6, 7). Many bacterial virulence factors must be secreted to facilitate successful host invasion. As soon as these products leave the cell, they are potentially exploitable by social “cheats” (3, 8, 9). If the production of virulence factors depends on cooperation, the virulence and epidemiology of pathogenic infections will be determined by the outcome of competition between cooperative and cheating strains (10–12).

Although the hypothesis that cooperation drives virulence has gained much theoretical attention, there is an absence of empirical support from natural host-parasite systems. Data from artificial microcosms have confirmed that several microbial traits are cooperative (3, 8, 9, 13, 14) and that these traits can have virulence consequences (15, 16). However, the extent to which cooperation drives virulence in natural host systems and the extent to which natural infection leads to the potential for social interactions remain unclear. Furthermore, previous work has involved careful management of both population structure (relatedness) and population dy-

namics. This is a simplification that obscures the extent to which social behaviors and population processes can interact. In natural systems, levels of cooperation are expected to influence population growth, and population-level processes (immigration, emigration, and differential growth) will determine the degree of mixing of cooperators and cheats, which in turn determines the relative fitness of cooperators (14, 17). It therefore remains to be seen whether the conditions that favor bacterial cooperation persist in the face of natural population dynamics.

Here, we examine the consequences of cooperation at the individual and population levels with a mixture of laboratory and field experiments in a natural host-pathogen system, involving the bacterium *Bacillus thuringiensis*

and larvae of the diamondback moth, *Plutella xylostella* (Fig. 1A). *B. thuringiensis* is a widespread specialist parasite of invertebrates (18). These bacteria produce diverse proteinaceous crystal toxins that form crystalline inclusions at sporulation, which determine virulence and host-range. After ingestion by an insect host, crystal (Cry) toxins are solubilized and bind to the midgut membrane, where they induce midgut cell death (19). The perforated midgut allows *B. thuringiensis* to invade the host, an essential process because access to the hemocoel and death are essential for effective replication of this pathogen (20). Cry toxin inclusions are substantial (Fig. 1B), representing ~25% of the total dry weight of *B. thuringiensis* at sporulation (21), and so their production is likely to be metabolically costly. The size of these inclusions ensures that spores of producers and nonproducers of toxin can be readily distinguished with standard microscopy. Cry toxins are produced in 25 to 75% of clones from natural isolates of *B. thuringiensis* and related strains, which suggests that both producers and nonproducers coexist stably in natural populations (22, 23). *B. thuringiensis* has been widely studied as a source of microbial pesticides, and Cry toxin genes have been incorporated into genetically modified crops.

We first tested the extent to which toxin production is a social trait within insect larval hosts under controlled laboratory conditions. If Cry toxin production is a social trait, then the benefit of toxin production will be shared with nonproducers, who avoid the cost of producing the toxin. We created toxin nonproducers by



Fig. 1. (A) Bagged plants at experimental field site on Wytham Farm, Oxfordshire. (B) Phase-contrast micrograph of cells from sporulated culture *B. thuringiensis* kurstaki, isolate HD-1 (ST8) just before lysis; parasporal protein crystals lie adjacent to oval spores and are marked with solid triangles. [Micrograph courtesy of B. A. Federici]

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curing them of Cry-toxin-bearing plasmids via culture at high temperature (24) (supplementary text), as competition between near-isogenic strains with and without these plasmids is likely to occur in natural populations (22). We inoculated *P. xylostella* with a controlled dose of spores within 24 hours, using a range of frequencies of toxin producers and nonproducers (24). Nonproducers were unable to infect the host without being coinoculated with producers (Fig. 2). As caterpillars were exposed to an increasing proportion of toxin producers, the proportion of successful infections increased rapidly, reaching an asymptote after ~30% producers (Fig. 2A). In these mixed infections, the proportions of nonproducers increased relative to the producers over the course of infection, which implied that their growth rates were higher because they avoided the costs of toxin production (Fig. 2B). This supports the hypothesis that toxin production is cooperative and that solubilized toxin in the larval midgut can be exploited by nonproducers to gain access to the hemolymph (21).

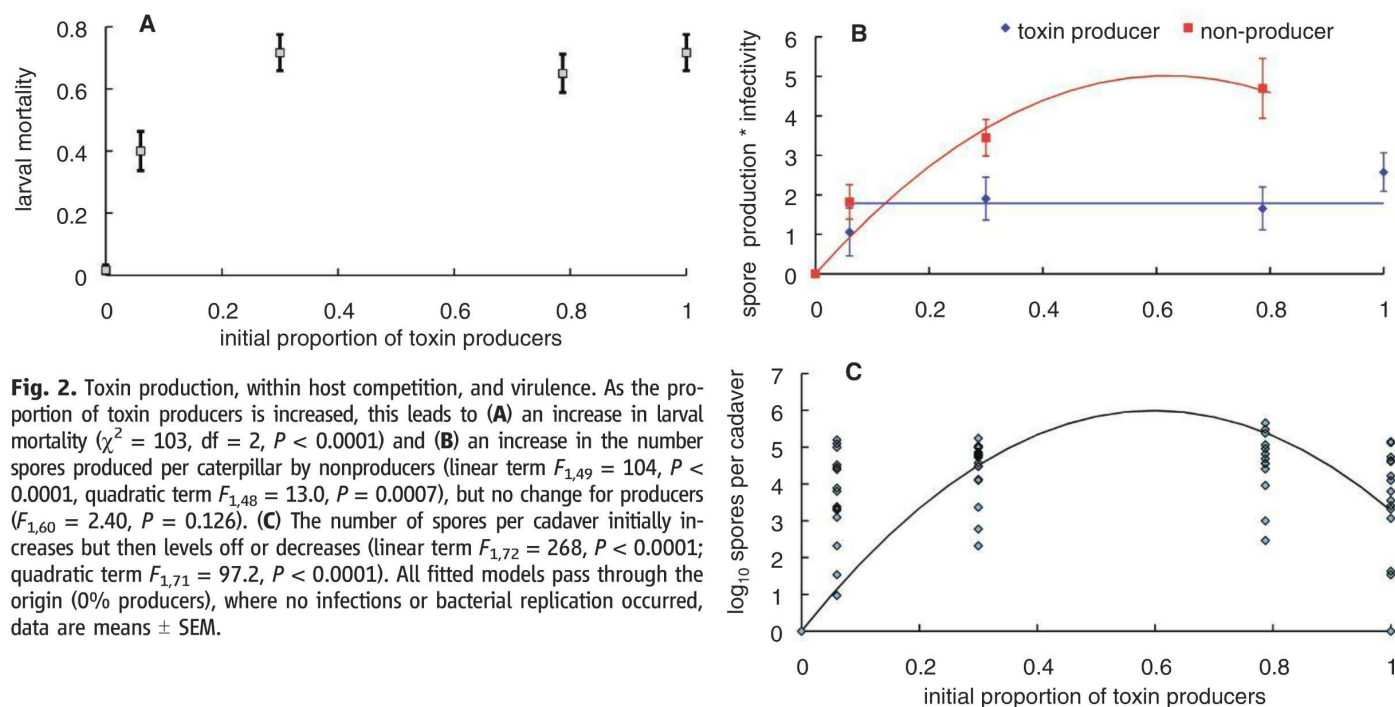
Our results show that selection on toxin production differs from that in other known social traits in bacteria. Increased production of bacterial extracellular factors, such as siderophores and elastases (3, 8, 9), leads to increased growth. In contrast, with Cry toxin production, once a bacterium has successfully invaded the hemocoel, there is no further benefit from extra toxin production, and so growth will be greater when there is a higher proportion of nonproducers, which avoid the cost of producing the toxin. This leads to a trade-off at the population level between the ability to infect caterpillars (which

requires producers) and growth in infected caterpillars (where nonproducers grow better), which can lead to greater population growth within hosts at an intermediate proportion of producers (Fig. 2, B and C). However, the key cost of toxin cheats in this pathogen system is in reduced transmission. The presence of cheats may not diminish population size within hosts, but they will reduce the overall toxin production in a cadaver and lower the probability of ongoing infections (Fig. 2A).

We examined how the social costs and benefits of toxin production play out under field conditions. Theory predicts that the relative fitness of nonproducers will depend on the extent to which they are able to coinfect hosts with toxin producers, which will depend on ecological factors, such as the density of bacteria, frequency of producers, and the extent to which producers are aggregated (14, 17). For example, higher population densities, a greater frequency of producers and less aggregation between patches will all make hosts more likely to ingest both producers and nonproducers and, hence, increase the extent to which nonproducers can exploit cooperators. We planted 204 six-week-old cabbages at Wytham Farm, near Oxford, UK, and bagged them with a fine net to exclude herbivores and their natural enemies (Fig. 1A). One week later, we added 35 third instar diamondback moth larvae to each plant. After a further 48 hours, we inoculated each plant with *B. thuringiensis* spores, with a factorial design that involved three density treatments and five frequency treatments of toxin producers (figs. S2 and S3) (24). We took two leaf samples per plant at time 0, and 14, 28, and 56 days after

spraying and recovered *B. thuringiensis* spores from saline leaf washes. This regime allowed larvae that have ingested spores to die and release new inoculum onto host plants and soil between sample dates (22). Insect populations crashed after day 28, and additional larvae ($n = 30$ per plant) were released at day 45.

We found that cooperation and virulence were determined by the way in which relative frequency and density affected the competitive dynamics of toxin producers and nonproducers (Fig. 3). When we examined across plants, nonproducers did better when at higher cell densities ($df = 9$, likelihood ratio = 4.71, $P = 0.030$) and when producers were more common [mixed-model analysis of variance (ANOVA), $df = 8$, likelihood ratio = 33.8, $P < 0.0001$]. This is expected given that these factors would make nonproducers more likely to coinfect hosts with producers. Furthermore, the nature of the frequency dependence was such that both types, producer and nonproducer, were able to increase in fitness when rare. We then analyzed frequency dependence within plants, examining plants for which we had reasonable abundance of *B. thuringiensis* over two consecutive time points, and found the same pattern. Considering changes in producer frequency from both 0 to 14 days and 14 to 28 days, we found that the relative fitness of nonproducers over this period was significantly negatively correlated with the starting proportion of nonproducers ($F_{1,31} = 32.7$, $P < 0.0001$) (Fig. 3B). It was noteworthy that the relative fitness of the nonproducers was >1.0 when rare and reached <1.0 when common, which showed again that both producers and nonproducers can invade when



rare and explains why both types are observed in natural populations.

Relatedness, or the spatial separation of producers and cheaters, is critical to the persistence

of cooperative traits (25). Typically, relatedness is inferred from neutral population genetic markers (26). Here, we were able to score relatedness directly at the *cry* toxin gene, because strains

expressing toxins are identifiable with light microscopy. Spatially aggregated populations of co-operators will emerge if plant colonization by *B. thuringiensis* is rare and there is fine-scale

Fig. 3. Frequency- and density-dependence in the field. **(A)** The proportion of toxin producers (arc-sine square root transformed) plotted against time over the first 14 days of the experiment. The data are divided according to the initial proportion of producers in experimentally applied *B. thuringiensis* (i.e., 0, 0.05, 0.5, 0.95, and 1.0) and the density of this inoculum (low or high). The relative fitness of producers can be inferred from the slopes of the fitted model; producers have higher relative fitness (positive or near zero slopes) when rare and at lower population densities. **(B)** Examining changes in the frequency of nonproducers over time, within plants, the relative fitness of nonproducers was lower when they were more common, considering changes from both 0 to 14 and 14 to 28 days. Cheats have equal fitness with producers when their fitness = 1. Each data point represents a single independent plant.

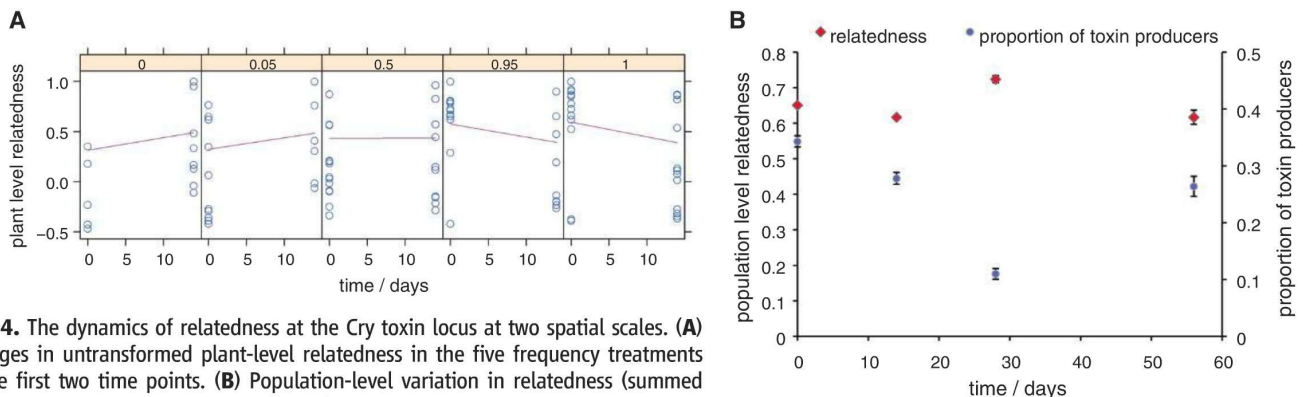
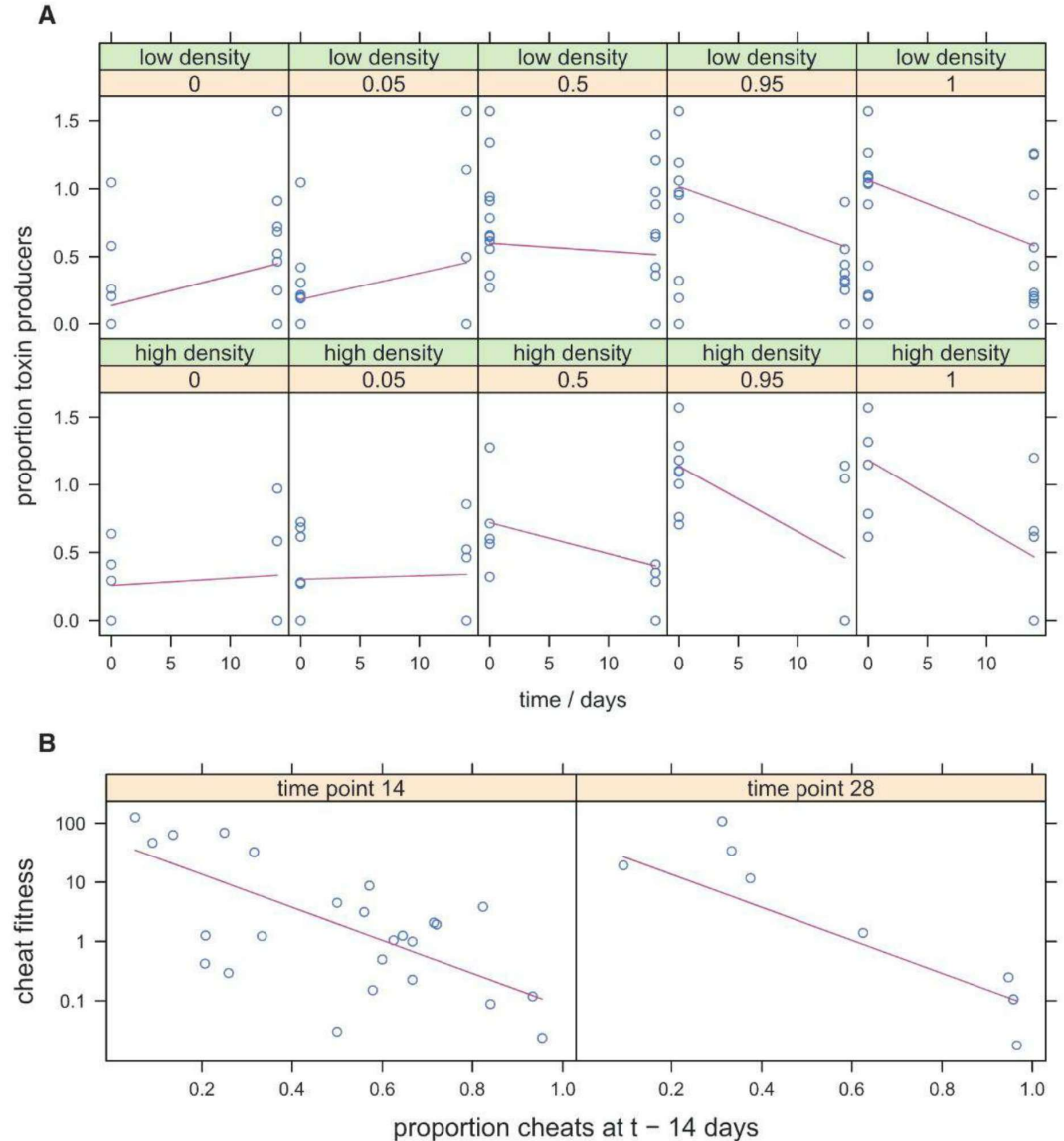


Fig. 4. The dynamics of relatedness at the *Cry* toxin locus at two spatial scales. **(A)** Changes in untransformed plant-level relatedness in the five frequency treatments at the first two time points. **(B)** Population-level variation in relatedness (summed across all plants in the experimental plot) and proportion of toxin producers: error bars for relatedness are 99% jackknifed confidence limits, calculated with $n = 125, 81, 84$, and 50 plants, for time points $0, 14, 28$, and 56 , respectively. Proportional data are means $\pm$ SEM for $n = 2335, 1863, 1097$, and 614 strains for time points $0, 14, 28$, and 56 .

competition between patches of microbes based on investment in toxin production. When we considered the first 14 days of the experiment, changes in relatedness tracked the changes in frequency of toxin producers, as might be expected from standard definitions of relatedness (supplementary materials), so that treatments with initially low frequencies of toxin producers had higher relatedness at time point 14 (treatments 0 and 0.05, Fig. 4, frequency*time interaction, likelihood ratio = 22.6, $P < 0.0001$). However, despite the rapid turnover in microbial populations at the plant level and wide fluctuation in the frequency of toxin producers between time points, relatedness at the level of the entire field plot remained high throughout the experiment (Fig. 4B). The highly aggregated distribution of bacteria on growing leaf tissue (supplementary materials and supplementary text) provides indirect support for our hypothesis of rare colonization. Evidence of the benefits of toxin production for local bacterial populations was found in the correlation between toxin production and bacterial density in the final time point of the field experiment (fig. S5; supplementary materials and supplementary text).

These experiments illustrate how cooperative, virulent bacteria and avirulent cheats compete and coexist in a natural environment. As social evolutionary theory predicts, nonproducing cheats can outcompete producers both within hosts and within local populations. Here, we have shown that cooperation can still be stable, because cheats drive down population density, and because cooperators do better when rare (negative frequency dependence) and at low population densities (negative density dependence). The rapidity with which toxin cheats can invade patches of cooperators is likely to have consequences for bacterial population dynamics. *B. thuringiensis*, unlike most insect pathogens, very rarely causes epidemics in the field (27, 28). The increased fitness of cheats at high densities and their ready availability in the soil mean that invasion of noninfectious cheats could rapidly curtail the direct host-host transmission required for an epidemic. In the field, *B. thuringiensis*-killed cadavers tend to fall into the soil soon after death (22), which suggests that most natural infections will occur indirectly after dispersal from a soil reservoir rather than from cadaver to larva during epidemics.

The more general implications of this work will depend on the prevalence of cooperative virulence among bacterial pathogens. However, a large number of essential virulence factors in important human pathogens are likely to be cooperative because they are exotoxins, which are necessarily secreted outside of the bacterial cell and are therefore potentially exploitable by cheats. These include anthrax toxins, diphtheria toxin, cholera toxin, Shiga toxin, *Clostridium* spp. exotoxins, pneumolysin, botulinum toxin, pertussis toxin, *Staphylococcus* alpha toxin, and tetanus toxin (29). In some instances (e.g., in

Vibrio cholerae) the ecological similarities with *B. thuringiensis* are striking; natural populations are composed of both toxin producers and nonproducers, toxin binding occurs on intestinal receptors and activates the release of host resources, and secreted toxins are encoded on mobile elements (30). Our results suggest that social interactions between toxin producers and nonproducers can explain the coexistence of virulent and avirulent bacteria and that sociality will influence the dynamics of virulence in natural populations.

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Supplementary Materials

www.sciencemag.org/cgi/content/full/337/6090/85/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S6
References (31–45)

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A Single Promoter Inversion Switches *Photorhabdus* Between Pathogenic and Mutualistic States

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Microbial populations stochastically generate variants with strikingly different properties, such as virulence or avirulence and antibiotic tolerance or sensitivity. *Photobacterium luminescens* bacteria have a variable life history in which they alternate between pathogens to a wide variety of insects and mutualists to their specific host nematodes. Here, we show that the *P. luminescens* pathogenic variant (P form) switches to a smaller-cell variant (M form) to initiate mutualism in host nematode intestines. A stochastic promoter inversion causes the switch between the two distinct forms. M-form cells are much smaller (one-seventh the volume), slower growing, and less bioluminescent than P-form cells; they are also avirulent and produce fewer secondary metabolites. Observations of form switching by individual cells in nematodes revealed that the M form persisted in maternal nematode intestines, were the first cells to colonize infective juvenile (IJ) offspring, and then switched to P form in the IJ intestine, which armed these nematodes for the next cycle of insect infection.

Pathogenic and mutualistic bacteria can exist in different states in their host to survive sudden environmental shifts such as antibiotic exposure or host immune activation (1, 2). *Photobacterium luminescens* bacteria are

bioluminescent symbionts of *Heterorhabditis bacteriophora* nematodes, and the two organisms (as a mutualistic pair) infect, kill, and reproduce inside insects. Nematodes in the infective juvenile (IJ) stage penetrate an insect host and

regurgitate their intestinal symbionts in the insect hemocoel (3), which leads to the death of the insect and the release of nutrients that support nematode reproduction (4). Inside the insect, the bacteria grow exponentially and secrete potent Tc and Mcf insecticidal toxins (5–7). The symbionts also produce crystalline inclusion proteins (Cips) with high levels of essential amino acids that are required for nematode reproduction, as well as antimicrobials that defend the insect cadaver from microbial competitors (8, 9). Nematode-bacterial associations were identified, and pulse-chase methodologies were performed (to limit symbionts) as described previously (10). The symbionts are maternally transmitted to IJ offspring developing inside the nematode's body (10). Mutualism is initiated when *Photorhabdus* phase variants express maternal adhesion (Mad) fimbriae and adhere to the nematode intestine (11). Fimbriae are proteinaceous surface filaments that function in bacterial cell adherence during host-cell colonization (12). Transmission proceeds through a series of steps involving adherence, invasion, and intracellular growth (10).

By visualizing individual cells inside nematode intestines, we discovered that *Photorhabdus* switched to a distinct small-cell form to initiate mutualism (M form), different from the insect pathogenic (P-form) cells that were only transiently present inside intestines (Fig. 1A and figs. S1 and S2). The M-form cells lacked visible CipA and CipB inclusions that were produced by the P form and are essential for nematode reproduction (8) (fig. S2). The M form grew as translucent small-colony variants (13) consisting of small cells, with opaque sectors of larger P-form cells erupting after 48 hours and eventually dominating the colony (fig. S3). Before our observations, the biological significance of small-colony variants was unknown, and the P form was considered the “wild type” because it is the predominant form isolated from entomopathogenic nematodes and infected insects, and it produces the antibiotics, bioluminescence, Cips, and insecticidal toxins normally associated with *Photorhabdus* bacteria (5–9, 14). Observing the development of this microbial symbiosis inside the nematodes revealed that the P form switches to the M form, which site-specifically colonizes the maternal nematode intestine to initiate mutualism.

Switching from P to M form correlated with promoter inversion from OFF to ON orientations

and, consequently, the expression of the Mad fimbrial locus. This switch enabled a minority variant population to selectively adhere to the posterior maternal intestine (fig. S4) (11). Inversion of the *madswitch* promoter occurring between two 36-base pair (bp) inverted repeats flanking 257 bp (Fig. 2) regulates *mad*, in an ON or OFF type of phase variation (15, 16). Cells stochastically express *mad*, which indicates that it is a highly mutable contingency locus (1). M-form cells transcribed *madA* from the *madswitch* when oriented ON and linked the M form with promoter inversion (fig. S4). We determined previously that P-form colonies aged up to 60 days in a Petri dish predominantly switched to the *madswitch* oriented ON (11). Viable cells isolated from these colonies then grew as the M form (fig. S5). However, the M form prevailed much sooner (4 days) in vivo after P-form infection of *Galleria mellonella* insect larvae, and this assay was subsequently used to examine form switching independent of mutualism (Fig. 2 and fig. S5). We developed a recombination methodology to manipulate the *Photorhabdus* genome (fig. S6). The original disruptions in *madA* and

madH predicted to encode the major subunit and usher-assembled fimbrial proteins, respectively, failed to generate the M form, which indicated that *mad* expression was required for M formation (Fig. 2). However, in-frame *madA* and *madH* deletion mutants still generated the M form, while retaining observable defects in nematode mutualism, including no detectable adhesion to the maternal nematode intestine or colonization of IJ nematodes. Taken together, these results suggest that the original mutations prevented M formation because of polarity on a distal regulator rather than on fimbriae structural genes.

One distal *mad* gene, *madJ*, was an attractive target as the possible cause of the M form because it encodes a homolog of CaiF (17) and of GrlA (18), transcriptional activators of enterohemorrhagic *Escherichia coli*. Deletion of *madJ* prevented M formation and severely compromised mutualism, which supported a functional role for MadJ as the regulatory output for M formation (Fig. 2, B and H, and Table 1). Half of the maternal nematodes associated with $\Delta madJ$ lacked detectable persistent bacteria; the other half had visibly fewer persistent bacteria and

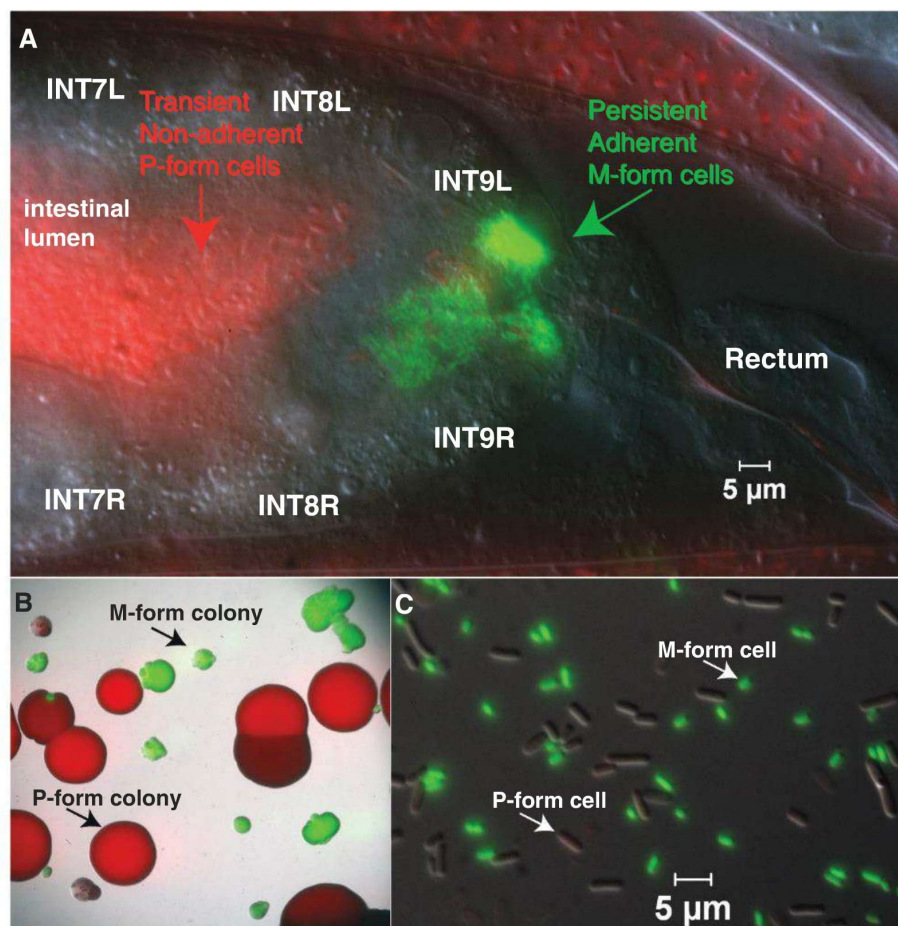


Fig. 1. *Photorhabdus* cells that initiate mutualism in the nematode intestine are small-cell variant M-form cells. (A) *Photorhabdus* cells that initiate mutualism (green) on the ninth intestinal ring cells, left and right, INT9L and INT9R, posterior intestinal cells and transients (red) are present throughout the lumen of the nematode. (B) M-form cells develop small colonies, and transient P-form cells develop large colonies. (C) Small colonies consist of primarily small cells, and large colonies consist of primarily larger cells.

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transmitted symbionts to only 21.5% of IJ progeny (Table 1). Attempts to complement the $\Delta madJ$ mutation by expressing *madJ* in trans from a broad host range plasmid were unsuccessful, because the plasmid was not maintained (see methods in the supplementary material). To further test the effect of *madswitch* inversion on mutualism and M formation, we deleted an invertase (i.e., a tyrosine-type site-specific recombinase) gene, *madR*, adjacent to the *madswitch*, but this failed to disrupt M formation or mutualism, which indicated that MadR is not involved in switching or that its function is redundant with another invertase that functions as the ON switch (Fig. 2, B and F). However, deleting a single orphan invertase gene, *madO* (*plu1991*), located 575 bp upstream of *fruB* (fig. S1), prevented M formation and mutualism (Fig. 2, B, G, and H, and Table 1), which indicated that MadO is required to switch the *madswitch* ON and to allow *madJ* expression. The expression levels of MadO could control the

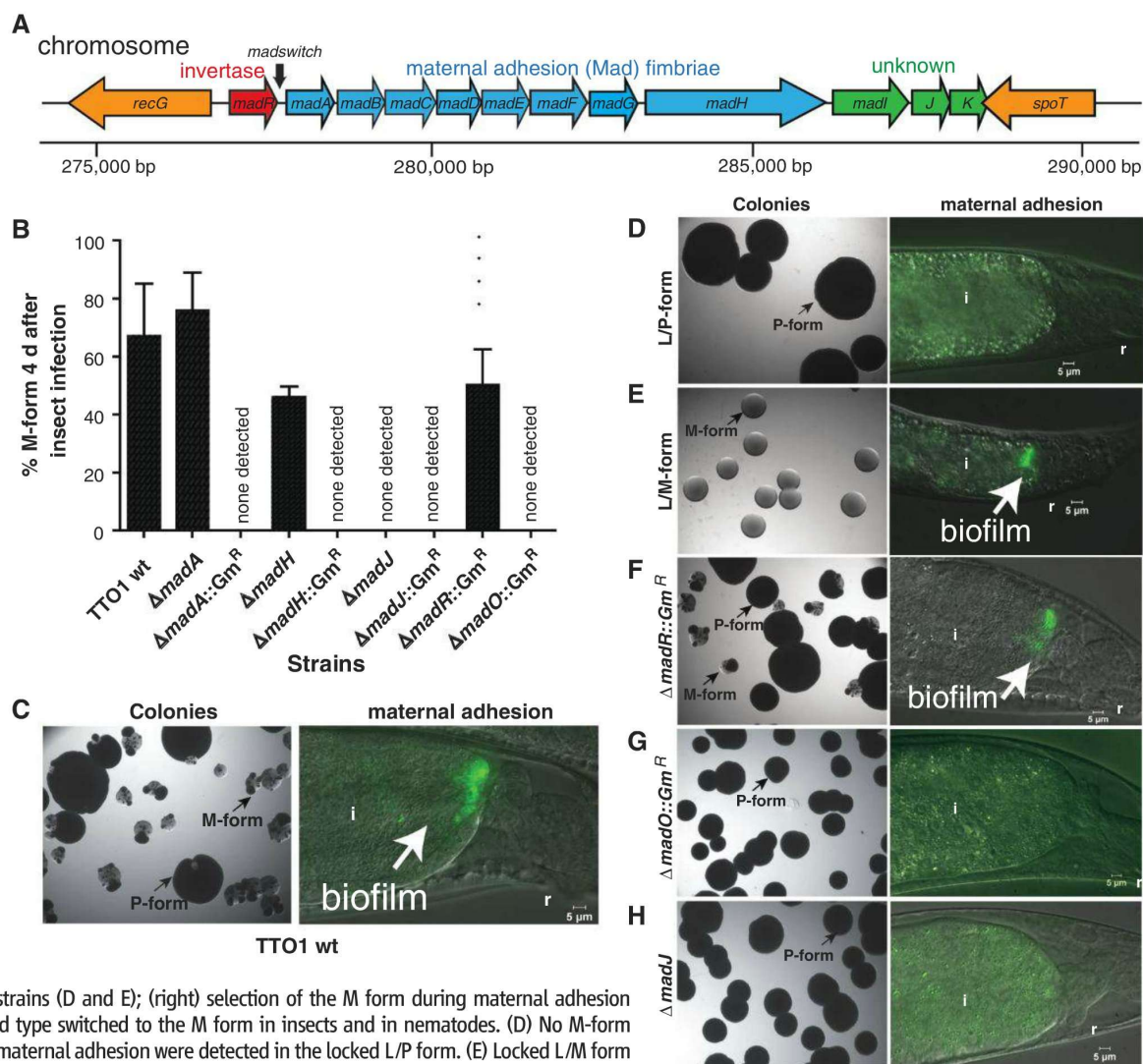
rate of promoter inversion from OFF to ON, or other factors may limit switching.

To test the effects of *madswitch* promoter inversion on phenotype switching, we genetically locked the *madswitch* to either the ON or OFF orientation (fig. S7). The *madswitch* was locked ON and OFF by recombining the switch in either orientation while deleting the upstream inverted repeat and *madR* (fig. S7). The *madswitch* reporter was similarly constructed, which resulted in *madA-FRT-gfp-madB*. M formation was assayed by injecting P-form cells into *Galleria mellonella* larvae, and then 4 days later determining the percentage of M-form colonies. Deleting the upstream inverted repeat in P-form cells locked the *madswitch* OFF and prevented the locked (L)/P-form (which failed to initiate mutualism) from being transmitted to IJ nematodes; cells that switched to the M-form phenotype were not detected (Fig. 2, B and D, and Table 1). Conversely, deleting the same repeat while inverting the *madswitch* ON

switched the P form to L/M-form colonies without P-form sectors (Fig. 2, B and E; fig. S3; and Table 1). All recombinants had the L/M-form phenotype; several of these were frozen, and one was used for further study. The L/M form initiated mutualism and was transmitted to IJ nematode offspring (Fig. 2 D and E, and Table 1). Thus, the orientation of the *madswitch* promoter determines the phenotype and life-style of *Photobacterium*.

Having locked M and P forms (fig. S7) enabled us to determine their many phenotypic differences. The M-form cells were shorter (length = 1.2 versus 4.4 μm), were smaller in diameter (0.8 versus 1.2 μm) and volume (0.7 versus 4.8 μm^3), and contained no visible Cips compared with the P form (fig. S3). The M form grew more slowly [$\mu = 0.09 \pm 0.003$ (SEM)] than the P form [$\mu = 0.13 \pm 0.02$ (SEM)] and was outgrown by the P form [competitive index = 106.3 ± 26.6 (SD)] (fig. S8) after 24 hours of growth at 28°C in lysogeny broth plus sodium pyruvate (LBP). The

Fig. 2. Expression of the *mad* fimbrial locus and *madO* are required for M formation. (A) Physical map of the *mad* locus required for maternal adhesion, which is an initial step of nematode mutualism. *madA* to *K* are co-transcribed and expressed by inversion of the *madswitch* promoter located between *madR* and *madA*. *madR* is predicted to encode a FimB-type site-specific recombinase (i.e., invertase). (B) Expression of *mad* is essential for M formation. The majority of T101 wild-type P-form cells switch to the M form 4 days after insect infection. Marked mutations (e.g., $\Delta madA::Gm^R$) of *madA*, *H*, *J*, and an orphan FimB-type recombinase *madO* failed to switch to the M form at detectable levels. $\Delta madR::Gm^R$ switched to the M form. In-frame deletions in *madA* and *madH*, but not *madJ*, led to a switch to the M form. Error bars represent standard deviation. (C to H) (Left) Colonies 4 days after P-form infection of insects, except the locked strains (D and E); (right) selection of the M form during maternal adhesion in nematodes. (C) T101 wild type switched to the M form in insects and in nematodes. (D) No M-form colonies ($N = 883$) and no maternal adhesion were detected in the locked L/P form. (E) Locked L/M form grew as M-form colonies ($N = 1656$) lacking sectors of the P form and adhering to the posterior nematode intestine. (F) Deleting the *MadR* invertase did not result in loss of switching to the M form in insects or nematodes. (G) Deleting the *MadO* invertase prevented switching to the M form in insects and nematodes. (H) Deleting *MadJ* resulted in no detectable M form in insects and 54% of maternal nematodes lacking adherent bacteria. i, intestine; r, rectum.



competitive disadvantage of the M form in co-culture was more than expected on the basis of the differences in growth rates. The M form produced less biofilm on polystyrene and was nonmotile (fig. S8). It also produced less bioluminescence, pigment, antibiotic, siderophore, and hemolysin (Fig. 3, A to E) and only minor amounts of rhabduscin, anthraquinone pigments,

and multipotent stilbenes (19). Stilbenes have been shown to act as antibiotics, insect phenoloxidase inhibitors, and nematode development signals (20, 21). All of these molecules were robustly produced by the P form (Fig. 3G). The M form produced more cinnamate, a precursor to stilbenes, after 48 hours of growth (fig. S9) likely because of less stilbene synthesis and

cinnamate hydrolysis (22, 23). IJ nematodes resumed development and reproduced less on the M form than on the P form (fig. S8). The M form had an altered capsule, absorbed less methylene blue and Congo red dyes, and exhibited a lower ability to reduce tetrazolium chloride dye (fig. S10). Finally, the M form was avirulent to insect *Galleria mellonella* larvae [time in which 50% of larvae die (LT_{50}) > 6 days, dose of ~100 cells] relative to the P form (LT_{50} < 2 days) (Fig. 3F). These radical differences likely reflect the dedicated function of each form (i.e., initiating nematode mutualism or insect pathogenicity).

Slow-growing small-colony variants and dormant persister cells are known to occur during chronic infections (24, 25). Because M forms emerge from prolonged aging of P-form colonies, we reasoned that the M form develops a higher incidence of persister cells (a high persister, *hip* phenotype). Indeed, the M form exhibited a *hip* phenotype, with >0.15% of cells tolerant to 1 μ g/ml ciprofloxacin after 3 hours of exposure, which was 49.3 times the 0.003% tolerance of P-form cells ($P = 0.01$) (Fig. 3H). The M form also exhibited more (8.7-fold, $P = 0.005$) tolerance to streptomycin aminoglycoside antibiotic (fig. S11). This degree of resistance provides further evidence that the M form is biased toward dormancy, which could be advantageous inside the nematode.

Table 1. M formation, maternal adhesion, and transmission of *Photorhabdus mad* mutants. Column 1, M-form cells in insects 4 days after injection of P-form cells. Column 2, adhesion in the intestines of maternal nematodes at 38 to 40 hours; $n = 40$ to 60 animals. Column 3, transmission in the intestines of 7-day-old IJ offspring; $n = 400$ to 500 animals. All values $\pm$ standard deviation of the mean; ND, none detected.

Strains	M form (%)	Maternal adhesion (%)	Percent transmission to IJs (%)
TT01 (wild type)	67.1 $\pm$ 18	98.4 $\pm$ 1.3	94.4 $\pm$ 2.9
L/P form	ND*	ND	ND
L/M form	100*	97.3 $\pm$ 1.6	92.1 $\pm$ 6.4
$\Delta madR::Gm^R$	50.2 $\pm$ 12.2	98.6 $\pm$ 1.9	90.6 $\pm$ 3.2
$\Delta madA::Gm^R$	ND	ND	ND
$\Delta madA$	75.8 $\pm$ 13.1	ND	ND
$\Delta madH::Gm^R$	ND	ND	ND
$\Delta madH$	46.0 $\pm$ 3.6	ND	ND
$\Delta madJ::Gm^R$	ND	41.4 $\pm$ 8.0	19.1 $\pm$ 3.5
$\Delta madJ$	ND	46.1 $\pm$ 8.0	21.5 $\pm$ 6.4
$\Delta madO::Gm^R$	ND	ND	ND

*Determined from colonies from an overnight liquid LBP culture; $n = 883$ for L/P form, $n = 1656$ for L/M form.

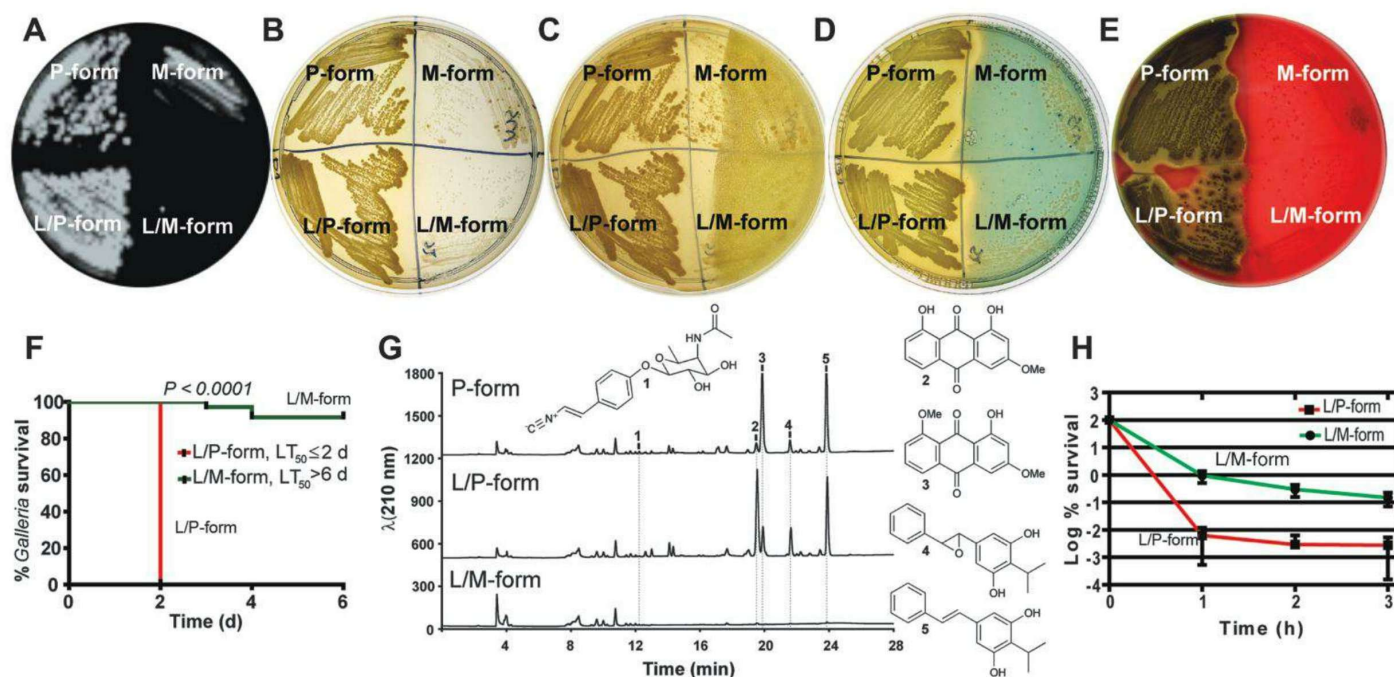


Fig. 3. Change in phenotypes, pathogenicity, secondary metabolite production, and persistence between P and M forms. (A) to (E) Quadrants on Petri dishes containing the P form (left), M form (right), unlocked forms (top), and locked forms (bottom). (A) Bioluminescence produced by the P form, which is absent in the M form (note that bioluminescence from P-form revertants is visible in the M form). (B) The P form is yellow and opaque, and the M form is unpigmented and transparent. (C) Antimicrobial activity produced at 48 hours of growth on LBP by the P form and not the M form against a soft-agar overlay containing *Micrococcus luteus* indicator bacteria. (D) Production

of siderophore iron-chelating activity by the P form and not the M form indicated as a zone of clearing as iron is removed from chrome azural S chelator. (E) Hemolytic activity on sheep blood agar produced by the P form but not the M form. (F) Virulence of the L/P form and not the L/M form after injection into *Galleria mellonella*. (G) Metabolite analysis detected the production of rhabduscin (1), two anthraquinone pigment molecules (2 and 3) and two hydroxystilbene molecules (4 and 5), which were mostly absent in the L/M form. (H) There are 50 times more persister cells tolerant to ciprofloxacin in the L/M form than in the L/P form.

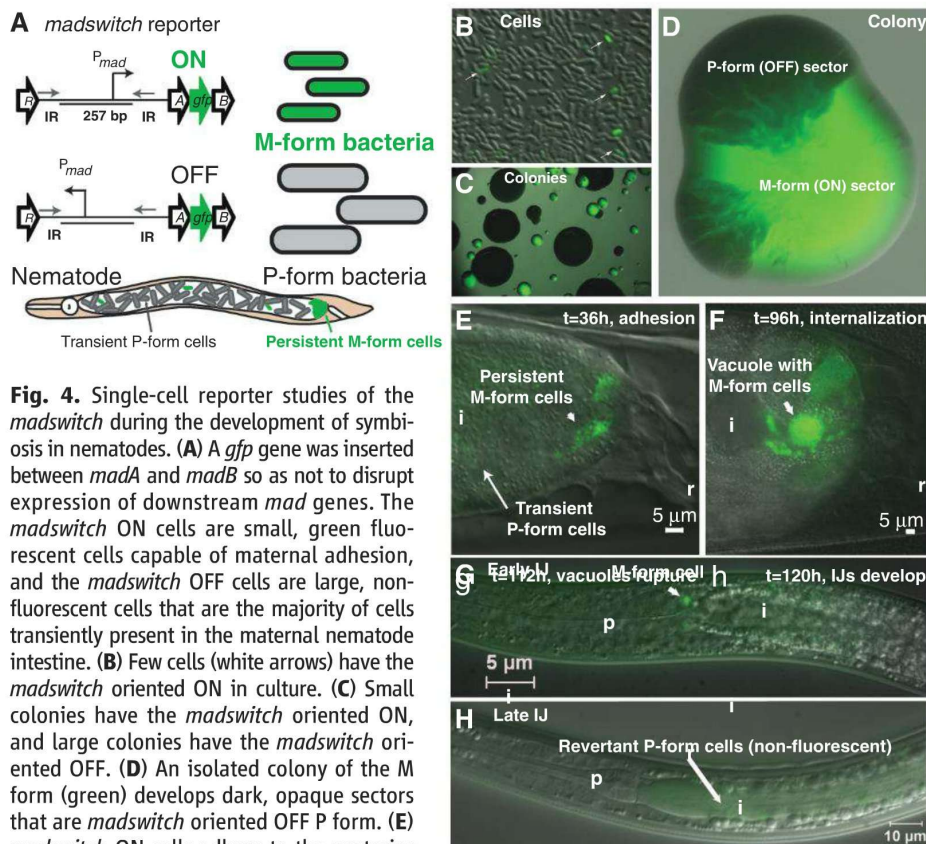


Fig. 4. Single-cell reporter studies of the *madswitch* during the development of symbiosis in nematodes. **(A)** A *gfp* gene was inserted between *madA* and *madB* so as not to disrupt expression of downstream *mad* genes. The *madswitch* ON cells are small, green fluorescent cells capable of maternal adhesion, and the *madswitch* OFF cells are large, non-fluorescent cells that are the majority of cells transiently present in the maternal nematode intestine. **(B)** Few cells (white arrows) have the *madswitch* oriented ON in culture. **(C)** Small colonies have the *madswitch* oriented ON, and large colonies have the *madswitch* oriented OFF. **(D)** An isolated colony of the M form (green) develops dark, opaque sectors that are *madswitch* oriented OFF P form. **(E)** *madswitch*-ON cells adhere to the posterior maternal nematode intestine, and most cells transiently present are not fluorescent, with the *madswitch* OFF. **(F)** Most adherent cells that invade and grow inside vacuoles of the rectal gland cells have the *madswitch* oriented ON. **(G)** One or two cells on or inside the pharyngeal intestinal valve cells have the *madswitch* oriented ON. **(H)** Seven days after the symbionts fully colonize the IJs, all cells are not fluorescent with the *madswitch* OFF, and again, the insect pathogenic P form is arming the nematode for insect infection. i, intestine; r, rectum; p, pharynx.

Furthermore, comparison of the transcriptomes of the locked M and P forms revealed that >10% of the genome, or 250 up-regulated genes (≥ 2 -fold, $P \leq 0.05$) and 265 down-regulated genes (≤ 0.5 -fold, $P \leq 0.05$), was differentially expressed in LBP at 24 hours and 28°C (tables S1 and S2). One locus highly expressed in the L/M form was that of clustered regularly interspaced short palindromic repeats (CRISPR)-associated sequences (CASs), which may function in gene silencing with other CAS-CRISPR sequences (26) (table S1). Other strongly up-regulated genes included *madA*, which was validated by quantitative reverse transcription polymerase chain reaction (fig. S12), and *hexA*, which is known to repress P-form phenotypes (27). Notable down-regulated genes were those encoding CipA and CipB (8), luciferase, PrtA protease (28), and Tc insecticidal toxins (5) (tables S1 and S2). The down-regulation of genes involved in heme and menaquinone biosynthesis (*hemD*, *hemE*, *menA*, *aroF*, and *aroQ*) indicates a reduced electron potential in the M form, like that of many clinical small-colony variants (24) (table S2).

In organisms that switch between phenotypic variants, analogous to cooperating and cheating,

the abundance of each type is a result of switching frequencies and variant fitness (29). The P to M form switching frequency (1.21×10^{-3} per cell per generation) was greater than the M to P form switching frequency (4.30×10^{-5} per cell per generation), which indicated that, although the P form switches to the M form 28 times more often than the reverse, the superior fitness of the P form enables it to remain dominant in culture (fig. S8). The higher fitness of the P form explains why M-form colonies are overtaken by the P form and why P-form colonies remain P-form colonies during laboratory growth (i.e., LBP, at 28°C, for 48 hours).

To visualize M formation at single-cell resolution, a green fluorescent protein *gfp* reporter was inserted between *madA* and *madB* (Fig. 4A). Few M-form cells were present in culture, and the resultant M-form colonies developed non-fluorescent sectors of P-form revertants (Fig. 4, B to D). Cells initiating nematode mutualism were green, whereas most cells transiently present in the nematode intestine were not (Fig. 4E). The M form prevailed during intracellular growth and the initial step of IJ colonization (Fig. 4, F and G). However, bacterial cells in the fully colonized IJ

intestine were not green and grew mostly as P-form colonies, which indicated that the IJs had become armed with the P form (Fig. 4, H and I, and fig. S13). Because the MadO invertase is required to flip the *madswitch* from OFF to ON, repressing MadO in IJ cells may favor MadR flipping of the *madswitch* from ON to OFF (fig. S1). Data supporting this model include the observation that 92.5% of colonies from IJs colonized by the $\Delta madR::Gm^R$ mutant were the M form, whereas only 0.29% were M form from IJs associated with P form (fig. S13).

Our examination of a multipartite interaction among bacteria, nematodes, and insects in a tractable model system provides key molecular insight into the drivers of phenotypic behaviors of *Photorhabdus* bacteria in their nematode and insect hosts. A stochastic promoter inversion switch controls the bacterial phenotypes, i.e., the M-form and P-form variants with dramatically different physiologies, required for initiating mutualistic or pathogenic life-styles, respectively. During initiation of nematode mutualism, most cells inside the maternal nematode intestine were P-form transients, but although fewer M-form cells persisted, they were preferentially transmitted to IJ offspring developing inside the maternal nematode. However, bacterial cells in fully colonized IJs switched back to the P form and armed these nematodes for insect infection. Chance promoter inversion appears to be an efficient mechanism in *Photorhabdus* to reversibly switch between conflict and cooperation with two different animal hosts.

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The microarray data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) (30) and are accessible through GEO Series accession no. GSE32088 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE32088>).

Supplementary Materials

www.sciencemag.org/cgi/content/full/337/6090/88/DC1
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Figs. S1 to S13
Tables S1 to S4
References (31–42)

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Identification and Functional Expression of the Mitochondrial Pyruvate Carrier

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The transport of pyruvate, the end product of glycolysis, into mitochondria is an essential process that provides the organelle with a major oxidative fuel. Although the existence of a specific mitochondrial pyruvate carrier (MPC) has been anticipated, its molecular identity remained unknown. We report that MPC is a heterocomplex formed by two members of a family of previously uncharacterized membrane proteins that are conserved from yeast to mammals. Members of the MPC family were found in the inner mitochondrial membrane, and yeast mutants lacking MPC proteins showed severe defects in mitochondrial pyruvate uptake. Coexpression of mouse MPC1 and MPC2 in *Lactococcus lactis* promoted transport of pyruvate across the membrane. These observations firmly establish these proteins as essential components of the MPC.

In the mitochondrion, pyruvate is converted into acetyl-coenzyme A (acetyl-CoA) by the pyruvate dehydrogenase (PDH) complex and participates in the synthesis of branched-chain amino acids (BCAAs) in yeast. Acetyl-CoA donates carbon atoms to the citric acid cycle and participates in the synthesis of octanoic acid, the precursor of lipoic acid (*l*). Lipoic acid is an essential cofactor of several multi-subunit complexes in the mitochondrial matrix, including PDH, α -ketoglutarate dehydrogenase (α -KDH), and the branched-chain keto acid dehydrogenase (BCKDH) (2). Import of pyruvate across the inner mitochondrial membrane (IMM) requires a specific carrier whose molecular identity has not been established (3). We have identified a family of membrane proteins (pfam UPF0041) whose members are necessary and sufficient for the transport of pyruvate into mitochondria. We have renamed this family the mitochondrial pyruvate carrier (MPC) family.

We previously identified MPC1 (formerly Brp44L) in a proteomic analysis of the IMM of mouse liver (4). MPC1 and its paralog MPC2 (formerly Brp44) have unknown function. Both are IMM proteins with three predicted transmembrane α -helices on the basis of secondary structure predictions and hydropathy profiling (fig. S1). They share sequence similarity with yeast Mpc1 (Ygl080w), Mpc2 (Yhr162w), and Mpc3 (Ygr243w) (fig. S1, C and D). We used *Saccharomyces cerevisiae* as a model organism to investigate the function of this family of proteins.

In yeast, Mpc1 localized in the IMM (fig. S2). Growth of the knockout mutants *mpc1Δ*, *mpc2Δ*, *mpc3Δ*, and *mpc2Δmpc3Δ* was normal on rich medium, either containing fermentable [yeast extract, peptone, and dextrose (YPD)] or nonfermentable carbon sources [Yeast extract, peptone, glycerol (YPG)] (Fig. 1A). In contrast, *mpc1Δ* and *mpc2Δ mpc3Δ* cells grew more slowly in amino acid-free medium [synthetic dextrose (SD)] than did the cells of the isogenic wild-type (WT) strain (Fig. 1A and fig. S3A). Deletion of *MPC2* alone led to a minor growth defect, whereas deletion of *MPC3* had no visible effect on growth. A similar phenotype was observed by (5).

Expression of WT *MPC1* restored growth of *mpc1Δ* cells (Fig. 1, B and C). Suppressor screens for genes that restored growth of *mpc1Δ* cells in SD identified only *MPC1* (supplementary text),

suggesting that its function cannot be complemented by other genes. Expression of either *MPC2* or *MPC3* restored growth of *mpc2Δmpc3Δ* cells but not that of cells lacking *MPC1* (Fig. 1, B and C). Thus, the growth of yeast in SD required a combination of Mpc1 with Mpc2 or Mpc3. We found that Mpc1 coimmunoprecipitated with Mpc2, suggesting that they form a heterocomplex (fig. S3B).

The growth defect in SD was relieved by addition of valine or leucine, with an additive effect of both amino acids (Fig. 1A). In contrast, addition of all amino acids except leucine and valine did not restore growth of the mutant strains (SC –V –L) (Fig. 1A). This finding prompted us to investigate the role of MPC proteins in the metabolism of valine and leucine. We assayed their decarboxylation by monitoring the release of ¹⁴CO₂ by cells grown in SD supplemented with either 1-¹⁴C valine or 1-¹⁴C leucine. Release of ¹⁴CO₂ by *mpc1Δ* cells was less than 2% of that in WT cells (Fig. 1D). A similar defect occurs in a *lpd1Δ* strain (Fig. 1E) (6), lacking a lipamide dehydrogenase essential for the function of the mitochondrial dehydrogenase complexes, PDH, α -KDH (7), and BCKDH (8). To test whether *mpc1Δ* cells also had dysfunctional PDH and α -KDH, we assessed their activities in mitochondria. The activity of these two complexes was impaired in mitochondria from *mpc1Δ* cells grown in SD (Fig. 1F), suggesting that the function of Mpc1 extends to several lipoyl-dependent complexes.

Lipoic acid is covalently attached to the E2 subunits of PDH (Lat1) and α -KDH (Kgd2), the E3-binding protein of PDH (Pdx1), and the H subunit of the glycine cleavage system (Gcv3) (9, 10). This modification can be readily assessed by means of protein immunoblotting with antibodies to lipoic acid (*l*). In rich medium, WT, *mpc1Δ*, *mpc2Δ*, *mpc3Δ*, and *mpc2Δmpc3Δ* cells had similar amounts of lipoylated complexes (Fig. 2A). However, when cells were grown in SD, lipoylated proteins were virtually absent from *mpc1Δ*, *mpc2Δ*, and *mpc2Δmpc3Δ* but not *mpc3Δ* cell lysates (Fig. 2A). Addition of valine or leucine, or both, to SD or expression of *MPC1* restored lipoylation in *mpc1Δ* (Fig. 2B and fig. S3C). The defect in lipoylation was correlated with decreased abundance of lipoic acid in *mpc1Δ* cells grown in SD (fig. S3D). In contrast, lipoic

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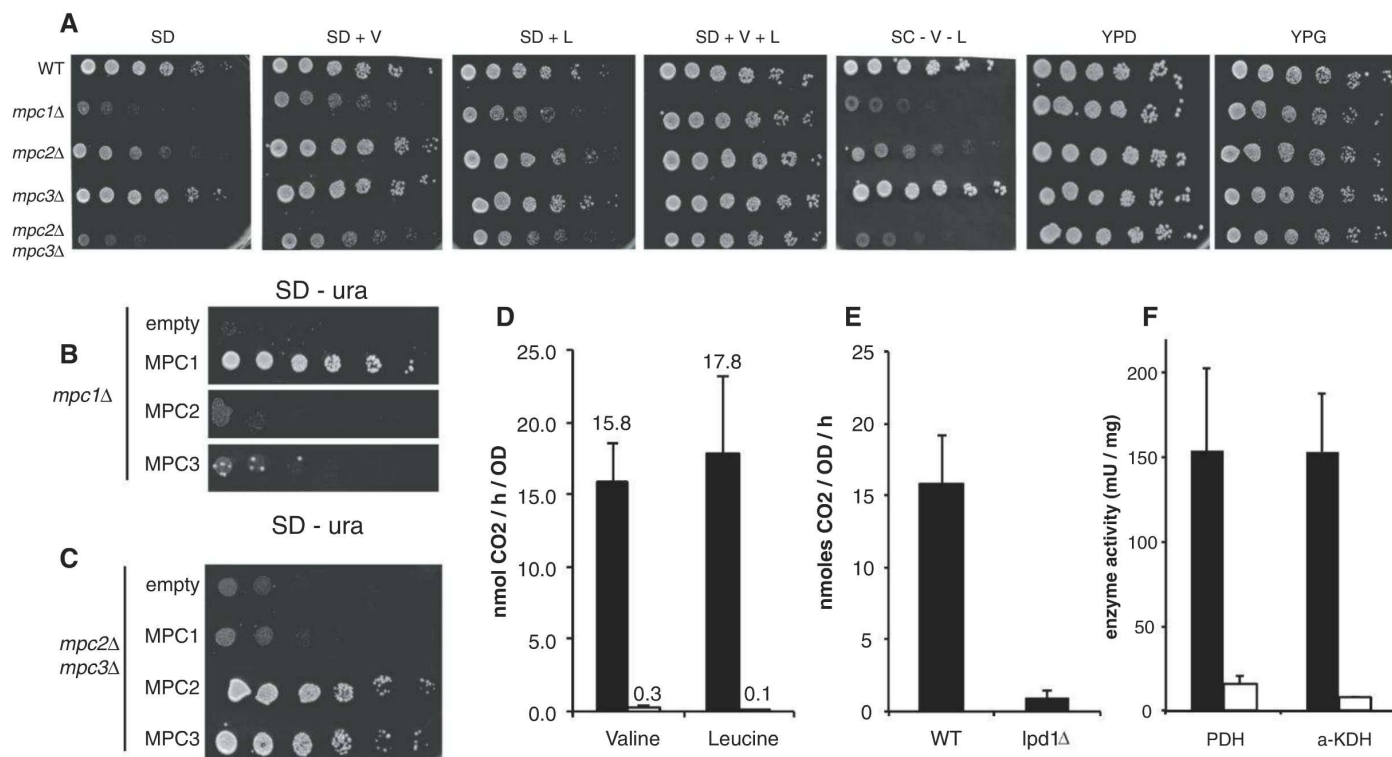


Fig. 1. Phenotypes of yeast deleted for MPC genes. **(A)** Spot assays of strains (as indicated) on media differing in amino acid composition or carbon source. The plates are representative of at least three independent experiments. **(B)** Spot assays of *mpc1Δ* cells transformed with p2U vector containing the indicated gene. **(C)** Spot assays of *mpc2Δmpc3Δ* cells transformed with p2U vector containing the indicated gene. **(D)** Valine or leucine

decarboxylation in WT (black bars) or *mpc1Δ* (white bars) cells. The means of six independent experiments are indicated above the bars $\pm$ SD. **(E)** Valine decarboxylation in the indicated strains. The mean of at least three independent experiments $\pm$ SD is shown. **(F)** PDH and α -KDH activities in WT (black bars) or *mpc1Δ* (white bars) mitochondria. Mean $\pm$ SD of three independent experiments for PDH and two independent experiments for α -KDH are shown.

acid was abundant in *mpc1Δ* cells grown in rich medium (fig. S3D). Thus, Mpc1 is essential for the production of lipoic acid and the function of lipoyl-dependent complexes only when cells are grown in SD, and this defect is relieved by the addition of valine and leucine.

We tested at which level the pathway leading to lipoic acid synthesis was defective in *mpc1Δ* cells grown in SD (fig. S4) (12). We excluded defects in the mitochondrial acetyl-CoA carboxylase HFA1, which converts acetyl-CoA into malonyl-CoA, or in the lipoic acid synthase Lip5, which converts octanoic acid into lipoic acid because cells lacking these enzymes cannot produce lipoic acid even when grown in rich medium (9, 13). In addition, no defect in the synthesis of octanoic acid from malonyl-CoA was detected in mitochondria isolated from *mpc1Δ* cells grown in SD medium (fig. S3E). Thus, the function of Mpc1 was likely to be upstream of acetyl-CoA (fig. S4). Because it is impossible to accurately measure amounts of acetyl-CoA in mitochondria, we tested the impact of reduced amount of mitochondrial acetyl-CoA on protein lipoylation by inhibiting PDH, the main source of acetyl-CoA from pyruvate. PDH-deficient cells (*pdx1Δ*) (10) were defective in protein lipoylation in SD, which was restored by addition of valine and leucine, similarly to *mpc1Δ* cells (Fig. 2B). However,

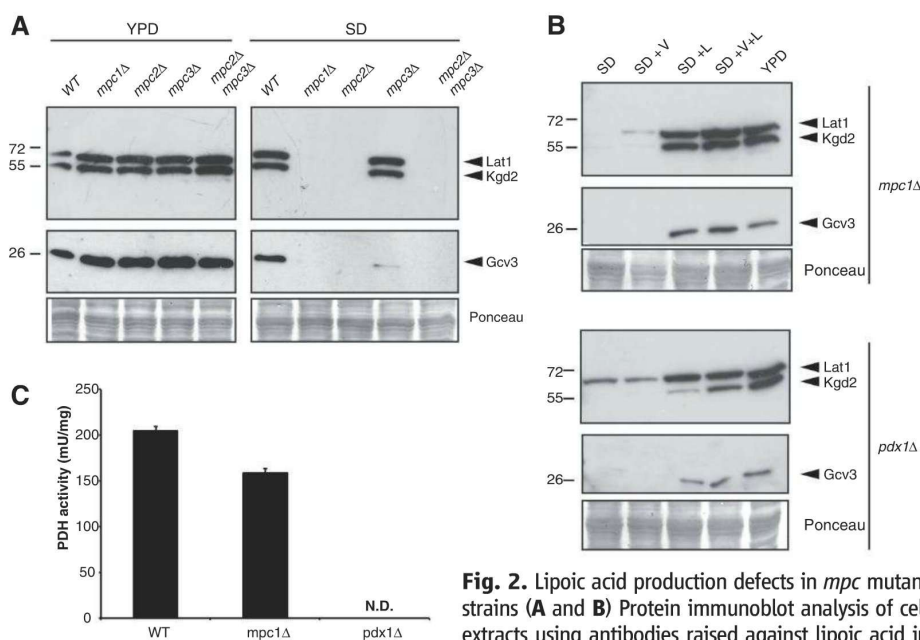


Fig. 2. Lipoic acid production defects in *mpc* mutant strains **(A and B)** Protein immunoblot analysis of cell extracts using antibodies raised against lipoic acid in strains and media as indicated. Ponceau staining is shown as loading control. The blots are representative of at least three independent experiments. **(C)** PDH activity of mitochondria isolated from the indicated strains grown in lactate medium. N.D., not detected. Data are expressed as the mean $\pm$ SD of three independent experiments.

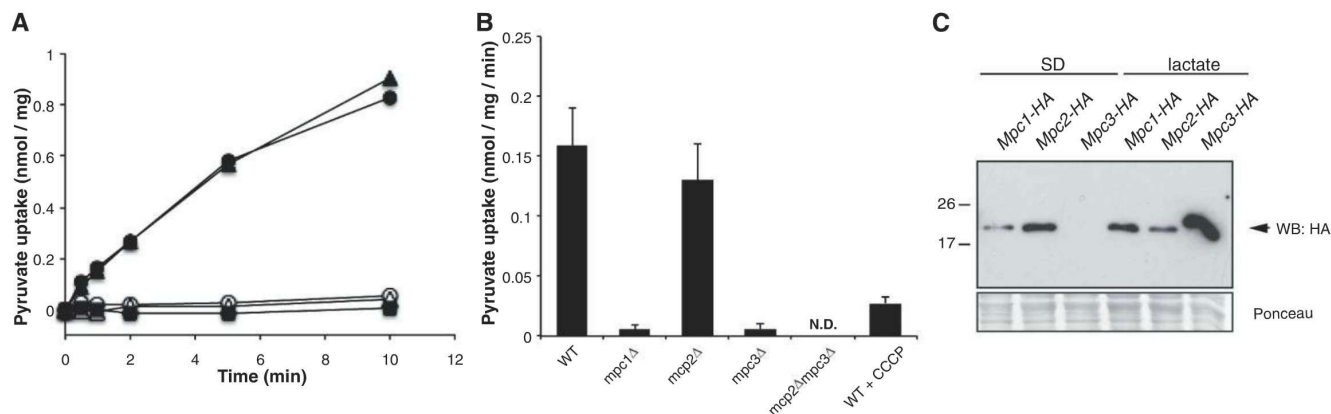


Fig. 3. Impaired import of pyruvate in *mpc* mutant strains. **(A)** Kinetics of pyruvate import into isolated mitochondria of WT (closed circles), *mpc1Δ* (open circles), *mpc2Δ* (closed triangles), *mpc3Δ* (open triangles), or *mpc2Δmpc3Δ* (squares) cells. Data are representative of at least three independent experiments. **(B)** Rate of pyruvate import calculated from the first 2 minutes of import.

Data are mean of at least three independent experiments $\pm$ SEM. N.D., not detected. **(C)** Protein immunoblot analysis of protein levels using genomically 3× hemagglutinin (HA)-tagged proteins in the indicated medium, probed with antibodies to HA. Ponceau staining is shown as a loading control. The blot is representative of three independent experiments.

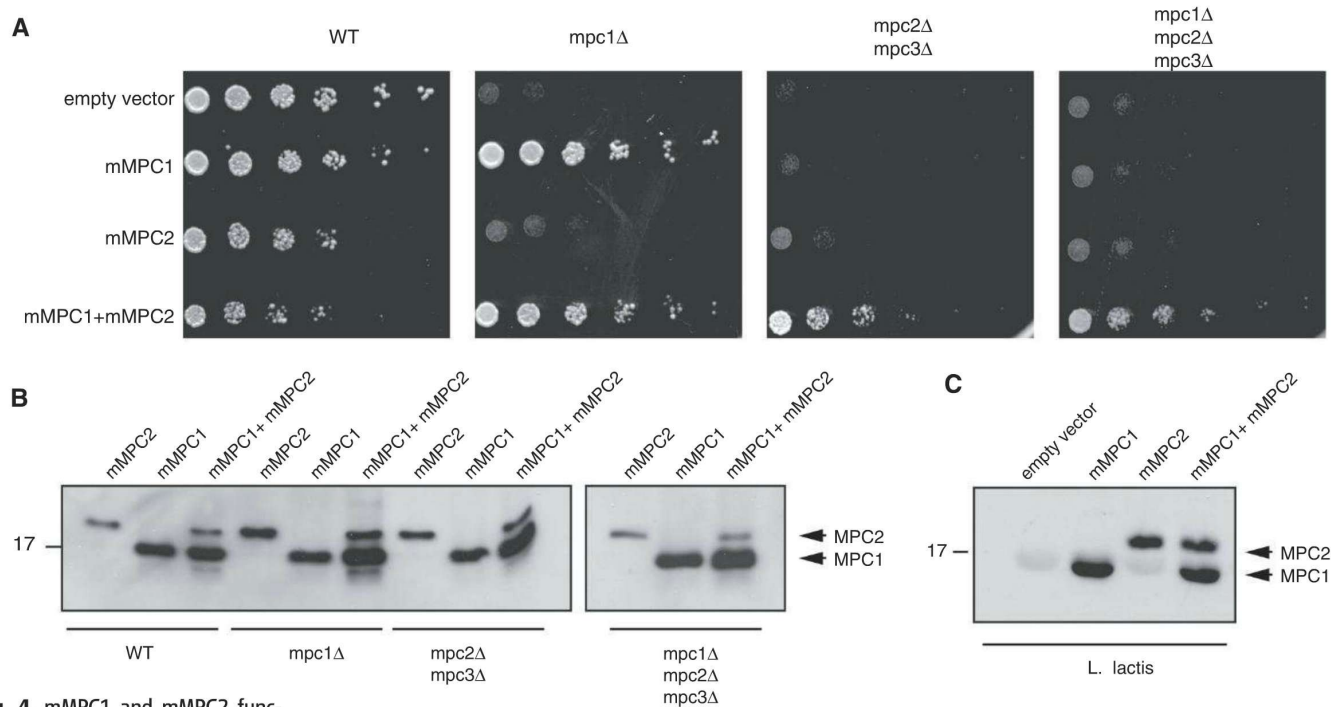


Fig. 4. mMPC1 and mMPC2 functionally reconstitute the pyruvate carrier in *L. lactis*. **(A)** Spot assay in amino acid-free medium of the indicated yeast strains transformed with an empty vector or expressing mMPC1 or mMPC2. **(B)** Protein immunoblot analysis of expression of the different proteins from strains shown in (A). **(C)** Protein immunoblotting of *L. lactis* strains showing expression of mMPC proteins. **(D)** Time course of pyruvate import in *L. lactis* strains with empty vector (diamonds), mMPC1 (circles), mMPC2 (triangles), or both (squares). The mean of three independent experiment $\pm$ SEM is shown. **(E)** Quantification of the import rate from the first 20 min of import in *L. lactis* strains expressing the indicated proteins. UK 5099 was at 50 μ M 2DG, 30 mM 2-deoxyglucose. * P < 0.05, two-tailed t test.

PDH activity was almost normal in *mpc1Δ* cells grown in rich medium [when the E2 subunit was lipoylated (Fig. 2C)]. Thus, the MPC proteins appeared to act upstream of PDH and may function in the transport of pyruvate into mitochondria. We therefore measured uptake of ^{14}C pyruvate in mitochondria isolated from WT, *mpc1Δ*, *mpc2Δ*, *mpc3Δ*, and *mpc2Δmpc3Δ* cells grown in lactate medium (Fig. 3A). The specificity of uptake was assessed by the use of UK5099, an inhibitor of the mitochondrial pyruvate carrier (14). Uptake of pyruvate in WT mitochondria was sensitive to the proton ionophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) (Fig. 3B). Mitochondria from *mpc1Δ* and *mpc2Δmpc3Δ* cells showed decreased pyruvate uptake (Fig. 3, A and B), despite a normal mitochondrial membrane potential (fig. S5). Surprisingly, deletion of *MPC3* alone impaired pyruvate uptake in mitochondria, whereas mitochondria from the *mpc2Δ* mutant transported pyruvate normally. Because this result did not correlate with the phenotypes of *mpc2Δ* and *mpc3Δ* single mutants grown in SD, we investigated the expression of Mpc2 and Mpc3 in SD and lactate media. In SD, yeast expressed mainly Mpc2, whereas in lactate medium, they mainly expressed Mpc3 (Fig. 3C). This expression pattern could be explained, at least in part, by the presence of binding sites for Gcn4 (a transcription factor activated by amino acid starvation) upstream of *MPC2* (15). This raises the possibility that under certain growth conditions, these two proteins might have specific, nonredundant functions.

We next assessed whether mouse MPC1 (mMPC1) and MPC2 (mMPC2) could restore growth of yeast cells lacking a functional pyruvate transporter (Fig. 4, A and B). mMPC1 alone restored growth of *mpc1Δ* cells, but mMPC2 failed to restore growth of the double-deletion strain of its orthologous genes *MPC2* and *MPC3*. However, growth of the triple-deletion strain *mpc1Δmpc2Δmpc3Δ* or of *mpc2Δmpc3Δ* cells was restored by coexpression of both mMPC1 and mMPC2 (Fig. 4A). Thus, mMPC1 and mMPC2 together functionally complement the absence of pyruvate transport. We next expressed mMPC1 and mMPC2, alone and in combination, in the bacterium *Lactococcus lactis* (Fig. 4C), which has been successfully used to express and characterize mitochondrial transporters (16). No pyruvate uptake was observed in bacteria expressing either protein alone compared with the empty vector control. However, a fourfold increase in pyruvate uptake was detected when mMPC1 and mMPC2 were coexpressed (Fig. 4, D and E). This uptake was sensitive to the mitochondrial pyruvate carrier inhibitor UK5099 and to 2-deoxyglucose, which collapses the proton electrochemical gradient (Fig. 4E) (17). Moreover, artificially increasing the membrane potential by lowering the pH in the import buffer from 7.2 to 6.2 significantly increased pyruvate uptake (two-tailed *t* test, $P < 0.05$) (Fig. 4E). Thus, coexpression of mMPC1 and mMPC2 in bacteria is

sufficient to allow import of pyruvate with similar properties to the mitochondrial pyruvate carrier (3). We therefore conclude that the mitochondrial pyruvate carrier is composed of Mpc1 and either Mpc2 or Mpc3 in yeast and of MPC1 and MPC2 in mammals.

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Supplementary Materials

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A Mitochondrial Pyruvate Carrier Required for Pyruvate Uptake in Yeast, *Drosophila*, and Humans

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Pyruvate constitutes a critical branch point in cellular carbon metabolism. We have identified two proteins, Mpc1 and Mpc2, as essential for mitochondrial pyruvate transport in yeast, *Drosophila*, and humans. Mpc1 and Mpc2 associate to form an ~150-kilodalton complex in the inner mitochondrial membrane. Yeast and *Drosophila* mutants lacking *MPC1* display impaired pyruvate metabolism, with an accumulation of upstream metabolites and a depletion of tricarboxylic acid cycle intermediates. Loss of yeast Mpc1 results in defective mitochondrial pyruvate uptake, and silencing of *MPC1* or *MPC2* in mammalian cells impairs pyruvate oxidation. A point mutation in *MPC1* provides resistance to a known inhibitor of the mitochondrial pyruvate carrier. Human genetic studies of three families with children suffering from lactic acidosis and hyperpyruvemia revealed a causal locus that mapped to *MPC1*, changing single amino acids that are conserved throughout eukaryotes. These data demonstrate that Mpc1 and Mpc2 form an essential part of the mitochondrial pyruvate carrier.

Pyruvate occupies a pivotal node in the regulation of carbon metabolism as it is the end product of glycolysis and a major substrate for the tricarboxylic acid (TCA) cycle in mitochondria. Pyruvate lies at the intersection of these catabolic pathways with anabolic pathways for lipid synthesis, amino acid biosynthesis, and gluconeogenesis. As a result, the failure to correctly partition carbon between these fates lies at the heart of the altered metabolism evident in diabetes, obesity, and cancer (1, 2). Owing to the fundamental importance of pyruvate, the

mitochondrial pyruvate carrier (MPC) has been studied extensively (3, 4). This included the discovery that α -cyanocinnamate analogs, such as UK-5099, act as specific and potent inhibitors of carrier activity (5). In spite of this characterization, however, the gene or genes that encode the mitochondrial pyruvate carrier remain unknown (6, 7).

As part of an ongoing effort to characterize mitochondrial proteins that are conserved through evolution, we initiated studies of the MPC protein family (originally designated BRP44 and BRP44L

in humans) (8). This family contains three members in *Saccharomyces cerevisiae*, encoded by *YGL080W*, *YHR162W*, and *YGR243W*, hereafter referred to as *MPC1*, *MPC2*, and *MPC3*, respectively. *Mpc2* and *Mpc3* are 79% identical in amino acid sequence and appear to be the product of a recent gene duplication event. *Mpc1*, *Mpc2*, and *Mpc3* colocalize with mitochondria (Fig. 1A and fig. S2A), consistent with published mitochondrial proteomic studies (9, 10). The mitochondrial localization of *Mpc1* and *Mpc2* was confirmed by biochemical fractionation (Fig. 1B). *Mpc1*, *Mpc2*, and *Mpc3* were enriched in mitochondrial membranes (fig. S2B), consistent with the presence of predicted transmembrane domains in their sequences (fig. S1). *Mpc1* and *Mpc2* were resistant to protease treatment unless the mitochondrial outer membrane was ruptured (Fig. 1B and fig. S2C), implying that they are embedded in the mitochondrial inner membrane. Chromatographic purification of tagged variants of *Mpc1* and *Mpc2*, followed by mass spectrometry, revealed that *Mpc2* and *Mpc3* were among the major interacting proteins of *Mpc1*, and *Mpc1* and *Mpc3* were among the major interacting proteins of *Mpc2* (table S1). Consistent with this, immunoprecipitation of tagged *Mpc1* copurified *Mpc2* and vice versa (Fig. 1C, lanes 3 and 4). In addition, *Mpc2* can interact with itself (Fig. 1C, lane 8), whereas an *Mpc1* homotypic interaction was not detected (Fig. 1C, lane 7). Blue native-polyacrylamide gel electrophoresis showed that both *Mpc1* and *Mpc2* migrated as part of an ~150-kD complex (fig. S2D). Loss of *Mpc2* prevented *Mpc1* from migrating in this complex, whereas an *mpc1Δ* strain showed elevated *Mpc2* complex formation (fig. S2E). We conclude that *Mpc1* and *Mpc2* form a multimeric complex embedded in the mitochondrial inner membrane, with *Mpc2* likely being the major structural subunit.

Mutant yeast strains were subjected to a variety of growth conditions. The *mpc1Δ* and *mpc2Δ* cells displayed mild growth defects on non-fermentable carbon sources like glycerol, with greater effects on glucose medium (fig. S3) and a strong growth defect in the absence of leucine (Fig. 1D). In contrast, *mpc3Δ* mutant displayed no apparent growth phenotypes. Yeast, *Drosophila*, or human *MPC1* orthologs, but not human *MPC2*, could rescue the *mpc1Δ* growth pheno-

type (Fig. 1E), indicating that *Mpc1* function is conserved through evolution.

To analyze the physiological function of MPCs in a multicellular animal, we extended our studies to the *Drosophila* ortholog of *MPC1* (*dMPC1*; encoded by *CG14290*), which also localized to mitochondria (fig. S4). Analogous to yeast *mpc1Δ* mutants, *dMPC1* mutants (fig. S5) were viable on standard food, but sensitive to a carbohydrate-only diet, with rapid lethality after transfer to a sucrose medium (Fig. 2A). Whereas the amount of adenosine 5'-triphosphate (ATP) was reduced in *dMPC1* mutants (Fig. 2C), along with triacylglycerol (TAG) and protein (fig. S6,

B and C), the amounts of carbohydrates were elevated, including the circulating sugar trehalose (Fig. 2D), glucose (Fig. 2E), fructose, and glycogen (fig. S6, A and D). These results suggest that *dMPC1* mutants are defective in carbohydrate metabolism and may consume stored fat and protein for energy. Consistent with this, the lethality of *dMPC1* mutants on the sugar diet was rescued by expression of the wild-type gene in tissues that depend heavily on glucose metabolism: the fat body, muscle, and neurons (Fig. 2B).

Metabolomic analyses revealed that the concentration of pyruvate was highly elevated, whereas TCA cycle intermediates were significantly

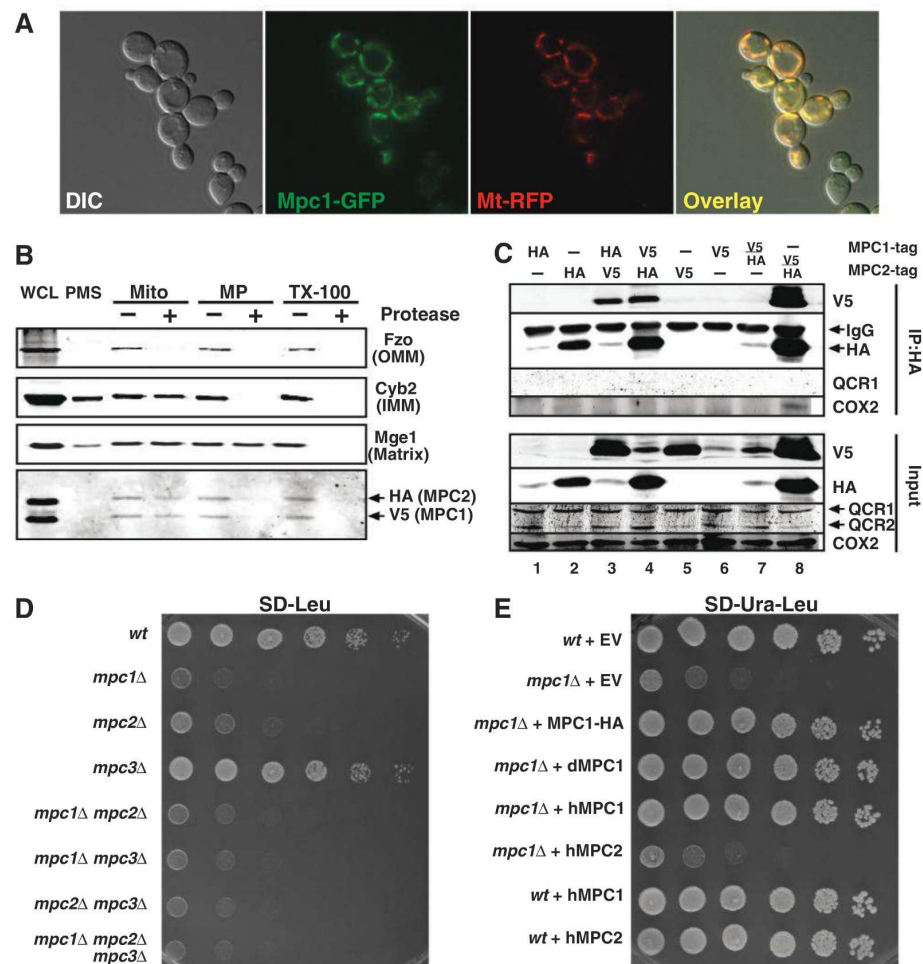


Fig. 1. *Mpc1* and *Mpc2* are evolutionarily conserved mitochondrial inner-membrane proteins. (A) *Mpc1* labeled with green fluorescent protein (*Mpc1*-GFP) and mitochondrial targeted red fluorescent protein (*MtRFP*) coexpressed in yeast cells. DIC, differential interference contrast. (B) Intact mitochondria, hypotonic-swollen mitoplasts, and TritonX-100-solubilized mitochondria from a strain expressing *Mpc1*-V5 and *Mpc2*-His₆/HA₂ with (+) or without (–) proteinase K incubation. An immunoblot of extracts using the indicated antibodies with the whole-cell lysate (WCL) and postmitochondrial supernatant (PMS) is shown. *Mge1*, *Cyb2*, and *Fzo1* are matrix, intermembrane space, and outer-membrane proteins, respectively. (C) Immunoprecipitations from mitochondrial extracts from *mpc1Δ mpc2Δ* cells expressing *Mpc1* and *Mpc2* tagged as indicated. Immunoblot of either immunoprecipitate (IP:HA) or input is shown (HA, hemagglutinin). *QCR1* and *2* (ubiquinol–cytochrome c reductase complex core protein 1 and 2) along with *Cox II* (cytochrome c oxidase subunit 2) are controls for the specificity of the immunoprecipitation. (D) Serial dilutions of the indicated yeast strains spotted on synthetic media lacking leucine and grown at 30°C for 24 hours. (E) Serial dilutions of indicated strains spotted on synthetic media lacking leucine and grown at 30°C for 48 hours. *wt*, wild type; EV, empty vector.

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depleted in *dMPC1* mutants on the sugar diet (Fig. 2F). Similarly, the amounts of glycine and serine, which can interconvert with glycolytic intermediates, were elevated in the mutants on the sugar diet (fig. S6E), whereas glutamate, aspartate, and proline, which can interconvert with TCA cycle intermediates, were depleted under these conditions (fig. S6F). Consistent with this, metabolomic analysis of *mpc1Δ* and *mpc2Δ* yeast mutants revealed elevated pyruvate concentrations (Fig. 3A), depletion of malate (fig. S7), depleted acetyl-coenzyme A (CoA), and elevated CoA concentrations (Fig. 3B). Taken together, these results suggest that *MPC1* mutants are unable to efficiently convert cytosolic pyruvate to mitochondrial acetyl-CoA to drive the TCA cycle and ATP production.

These phenotypes could arise from either a defect in mitochondrial pyruvate uptake or the conversion of mitochondrial pyruvate into acetyl-CoA by the pyruvate dehydrogenase (PDH) complex. Yeast lacking *Mpc1*, however, had nearly wild-type PDH activity, unlike the strong decrease seen in *pda1Δ* mutants (Fig. 3C), which lack PDH function (11). A decrease in PDH activity also does not explain the growth defect of *mpc1Δ* mutants, which is more severe than that of the *pda1Δ* mutant (fig. S8). However, combining the *mpc1Δ* allele with a deletion for *mae1*, which encodes a malic enzyme that converts malate to pyruvate in the mitochondrial matrix (12), revealed a profound growth defect on glucose medium that was completely rescued by plasmid expression of either *MAE1* or *MPC1* (Fig. 3D). Notably, mitochondria from the *mpc1Δ* mutant displayed almost no uptake of ^{14}C -pyruvate, which could be fully rescued by plasmid expression of wild-type *MPC1* (Fig. 3E). Moreover, *Mpc1* appears to be a key target for UK-5099, which is an inhibitor of the mitochondrial pyruvate carrier (5). The *mae1Δ mpc1Δ* double mutant displayed reduced growth on glucose medium lacking leucine, and this phenotype could be effectively rescued by transgenic expression of wild-type *MPC1* in the absence, but not the presence, of UK-5099 (Fig. 3F). By screening for *MPC1* mutants that could grow in the presence of UK-5099, we recovered an Asp¹¹⁸→Gly (D118G) substitution in *Mpc1* that conferred UK-5099 resistance (Fig. 3F). Moreover, whereas ^{14}C -pyruvate uptake into mitochondria expressing wild-type *MPC1* was almost completely inhibited by UK-5099, efficient pyruvate uptake that is resistant to UK-5099 was recovered upon expression of *MPC1-D118G* (Fig. 3G). We conclude that *Mpc1* is a key component of the mitochondrial pyruvate carrier that corresponds to the activity studied for decades by Halestrap and others (5, 13).

Depletion of *MPC1* in mouse embryonic fibroblasts (fig. S9A) caused a modest decrease in pyruvate-driven oxygen consumption under basal conditions, and a stronger reduction in the presence of carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), which stimulates maximal respiration (Fig. 4A). Similar results were also seen upon

silencing *MPC2* (Fig. 4B and fig. S9A). This suppression of pyruvate oxidation, which occurred without affecting components of the oxidative phosphorylation machinery (fig. S9, B and C), suggests that mammalian *Mpc1* and *Mpc2* mediate mitochondrial pyruvate uptake in a manner similar to that seen in yeast and *Drosophila*.

We have previously described a French-Algerian family with two offspring that exhibited a devastating defect in mitochondrial pyruvate oxidation (14) (Fig. 4C, family 1). We subsequently discovered two additional families, each with one affected child who displayed a similar, but less severe, phenotype (Fig. 4C, families 2 and 3). Linkage analysis and homozygosity mapping allowed us to focus on one candidate region on chromosome 6 (163,607,637 to 166,842,083, GRCh37/hg19). This interval contained 10 potential candidate genes: *PACRG*, *QKI*, *C6orf118*, *PDE10A*, *SDIM1*, *T*, *PRR18*, *SFT2D1*, *RPS6KA2*, and *BRP44L*, which is the human *MPC1*. DNA sequencing of the exons and intron/exon boundaries of the *MPC1* gene in fibroblasts from the affected patients in families 2 and 3 revealed the

same molecular lesion, c.236T→A, causing a predicted p.Leu⁷⁹→His (L79H) alteration (Fig. 4D). Analysis of DNA from family 1 revealed a distinct sequence change, c.289C→T, which resulted in a predicted p.Arg⁹⁷→Trp (R97W) mutation (Fig. 4D). Both of the affected residues are conserved through evolution between *MPC1* orthologs, and Arg⁹⁷ is conserved among both *MPC1* and *MPC2* orthologs (fig. S1).

Cells from the affected individuals in families 1 and 2 exhibited impaired basal and FCCP-stimulated pyruvate oxidation (Fig. 4E), whereas glutamine-driven oxygen consumption was normal or elevated, demonstrating that they have not acquired a generalized impairment of mitochondrial respiration (Fig. 4E). As expected, expression of wild-type human *MPC1* in the cells from family 2 (Fig. 4F) or family 1 (Fig. 4G) either completely or partially rescued the defect in FCCP-induced pyruvate oxidation. Moreover, expression of the *MPC1-Leu79His* allele was less effective at suppressing the yeast *mpc1Δ* growth defect relative to wild-type human *MPC1* (Fig. 4H), and the stronger *MPC1-Arg97Trp* allele was

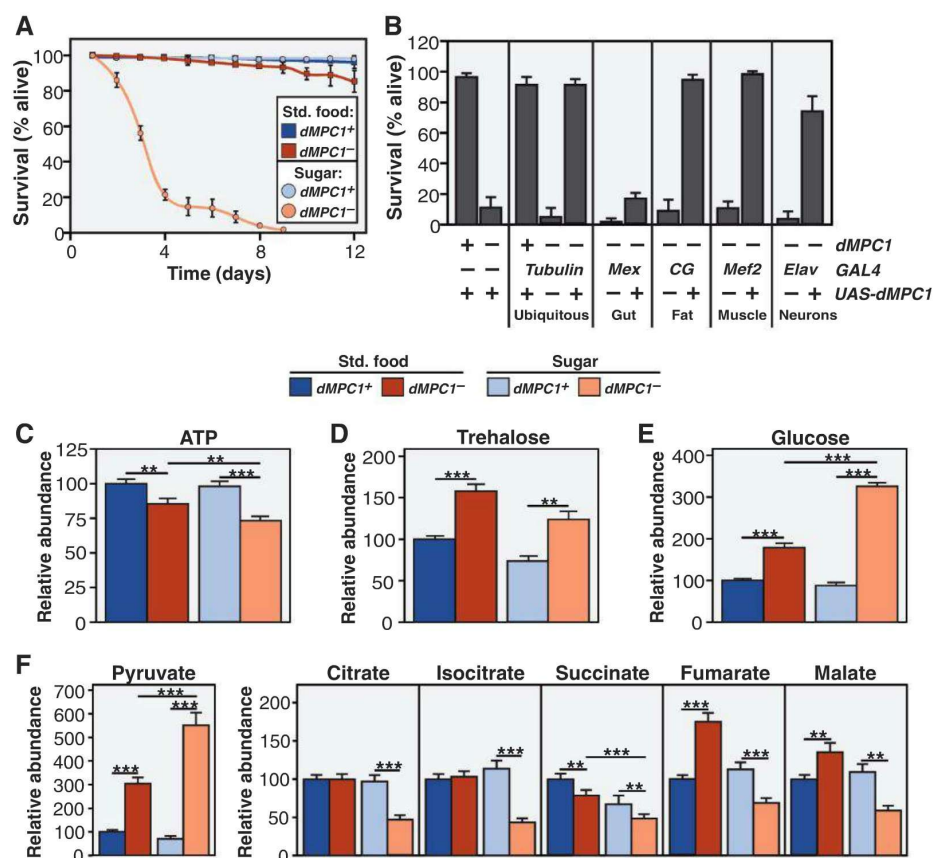


Fig. 2. *dMPC1* is required for pyruvate metabolism in *Drosophila*. (A) Percentage of living control (*dMPC1*⁺) or *dMPC1* mutant (*dMPC1*⁻) flies after transfer to standard laboratory medium (std. food) or to media containing only sugar. (B) Percentage of living *dMPC1*⁺ or *dMPC1*⁻ flies carrying the indicated GAL4 and UAS transgenes on sugar media after 8 days. (C to E) Relative concentration of ATP (C), trehalose (D), and glucose (E) in extracts from *dMPC1*⁺ or *dMPC1*⁻ flies on the indicated diet after either 2 days (D and E) or 3 days (C). (F) Relative abundance of pyruvate and TCA cycle intermediates in *dMPC1*⁺ or *dMPC1*⁻ flies after 2 days on the indicated diet as measured by gas chromatography–mass spectrometry. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 (Student's *t* test). Data are shown as mean ± SEM.

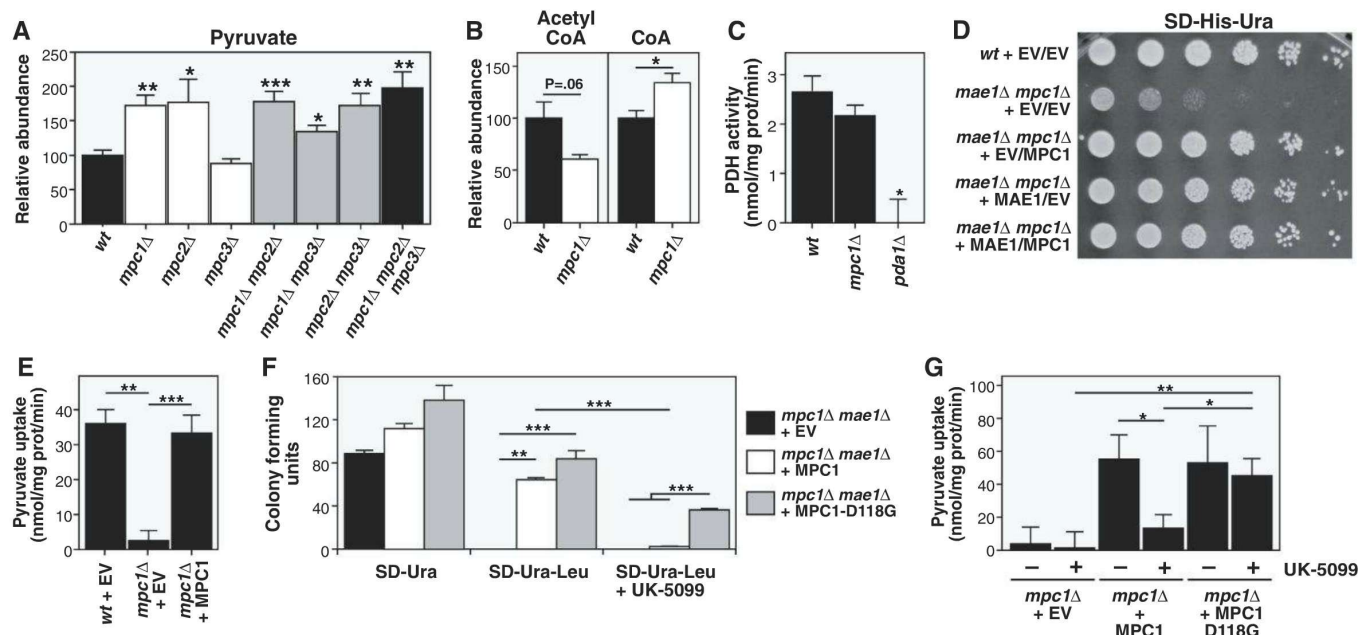
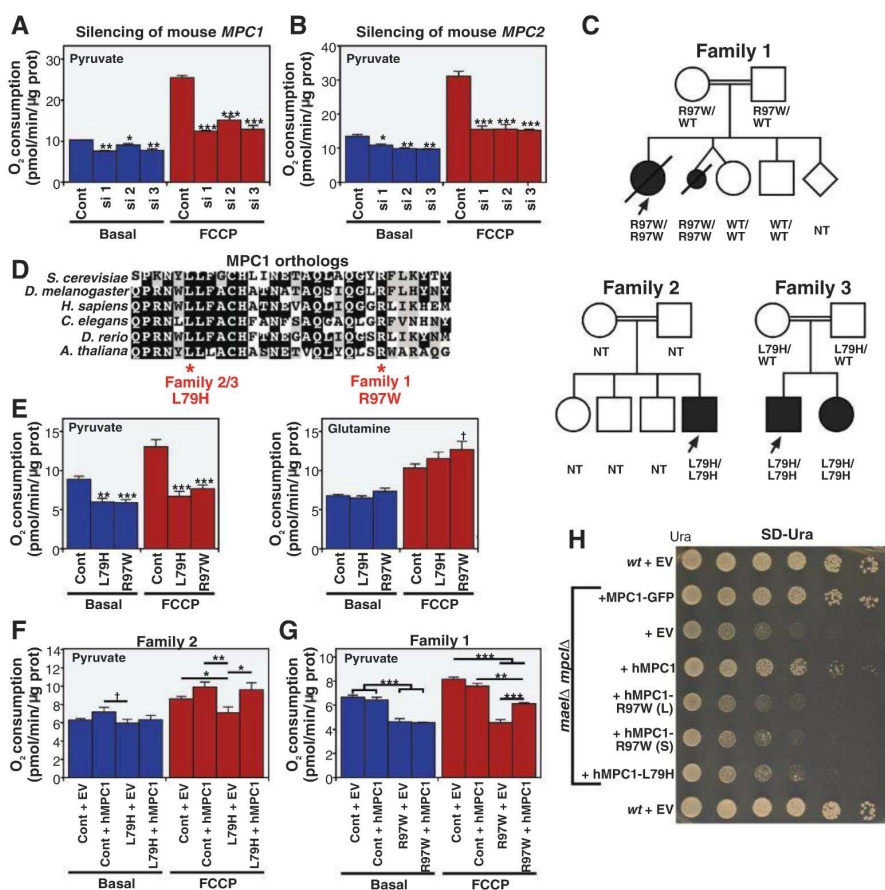


Fig. 3. *MPC1* is required for mitochondrial pyruvate uptake. (A) Relative abundance of pyruvate in the indicated strains. *P* values relative to *wt*. (B) Relative abundance of acetyl-CoA and CoA in the indicated strains. (C) Mitochondrial pyruvate dehydrogenase activity in the indicated strains. *P* value relative to *wt* and *mpc1Δ*. (D) Serial dilutions of the indicated strain on glucose medium grown at 30°C for 48 hours. (E) Uptake of ¹⁴C-pyruvate into mitochondria purified from either *wt* or *mpc1Δ* cells containing the indi-

cated plasmid. *P* value relative to *wt* + EV and *mpc1Δ* + MPC1. (F) *Mae1Δ mpc1Δ* cells transformed with the indicated plasmid and plated on media containing or lacking combinations of leucine or UK-5099. (G) Uptake of ¹⁴C-pyruvate into mitochondria isolated from the *mpc1Δ* strain containing the indicated plasmid in the presence or absence of UK-5099. ****P* < 0.001, ***P* < 0.01, **P* < 0.05; NS, not significant (Student's *t* test). Data are shown as mean ± SEM.

Fig. 4. Mammalian *MPC1* and *MPC2* are required for normal pyruvate metabolism. (A and B) Pyruvate-driven respiration in mouse embryonic fibroblasts under basal and FCCP-stimulated conditions in cells transfected with either control (Cont) small interfering RNAs (siRNAs) or three different siRNAs (si 1–3) targeted to either *MPC1* (A) or *MPC2* (B). *P* values relative to control. (C) Pedigrees of families 1, 2, and 3. Circles indicate females; squares, males; and diamonds, unknown sex. Black indicates deceased and white, living. Arrows mark individuals from whom fibroblasts were obtained. (D) The protein region of MPC1 containing the predicted amino acid substitutions from all three families aligned by ClustalW. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Homo sapiens*, *Caenorhabditis elegans*, *Danio rerio*, *Arabidopsis thaliana*. (E) Pyruvate- (left) and glutamine- (right) supported respiration of fibroblasts harboring either L79H or R97W *MPC1* mutations. (F) Pyruvate-supported respiration of either a control or an L79H patient cell line after transduction with the indicated vector. (G) Pyruvate-supported respiration of either a control or an R97W patient cell line after transduction with the indicated vector. (H) Serial dilutions of *wt* or *mae1Δ mpc1Δ* yeast strains carrying the indicated plasmid grown on medium lacking uracil for plasmid selection at 30°C for 40 hours. Both long (L) and short (S) forms of R97W were used (with or without exon 4). ****P* < 0.001, ***P* < 0.01, **P* < 0.05, †*P* < 0.10; NS, not significant (Student's *t* test). Data are shown as mean ± SEM.



essentially inactive (Fig. 4H), suggesting that *MPC1* function is evolutionarily conserved from yeast to humans.

The data presented here demonstrate that the Mpc1-Mpc2 complex is an essential component of the mitochondrial pyruvate carrier in yeast, flies, and mammals. This is consistent with experiments performed in rat liver, heart, and castor beans, which implicated proteins of 12 to 15 kD in mitochondrial pyruvate uptake (15)—similar to the molecular masses of Mpc1 (15 kD), Mpc2 (14 kD), and Mpc3 (16 kD). Although these individual sizes are relatively small, Mpc1 and Mpc2 form a complex of ~150 kD, suggesting that an oligomeric structure mediates pyruvate transport. The demonstration that Mpc1 and Mpc2 are sufficient to promote pyruvate uptake in a heterologous system provides further evidence that they constitute an essential pyruvate transporter (16). Finally, the degree to which carbohydrates are imported into mitochondria and converted into acetyl-CoA is a critical step in normal glucose oxidation as well as the onset of diabetes, obesity, and cancer. Thus, like PDH, which is controlled by allosteric and posttranslational modification (17), the mitochondrial import of pyruvate is likely to be precisely regulated (18, 19). The identification of Mpc1 and Mpc2 as critical for

mitochondrial pyruvate transport provides a new framework for understanding this level of metabolic control, as well as new directions for potential therapeutic intervention.

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Supplementary Materials

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An Abundance of Rare Functional Variants in 202 Drug Target Genes Sequenced in 14,002 People

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Rare genetic variants contribute to complex disease risk; however, the abundance of rare variants in human populations remains unknown. We explored this spectrum of variation by sequencing 202 genes encoding drug targets in 14,002 individuals. We find rare variants are abundant (1 every 17 bases) and geographically localized, so that even with large sample sizes, rare variant catalogs will be largely incomplete. We used the observed patterns of variation to estimate population growth parameters, the proportion of variants in a given frequency class that are putatively deleterious, and mutation rates for each gene. We conclude that because of rapid population growth and weak purifying selection, human populations harbor an abundance of rare variants, many of which are deleterious and have relevance to understanding disease risk.

Understanding the genetic contribution to human disease requires knowledge of the abundance and distribution of functional genetic diversity within and among populations. The “common-disease rare-variant” hypothesis posits that variants affecting health are under purifying selection and thus should be found only at low frequencies in human populations (1–3). This hypothesis has become

increasingly credible because very large genome-wide association studies of common variants have explained only a fraction of the known heritability of most traits (4, 5). Investigating the role of rare variants for complex trait mapping has led to tests that aggregate rare variants (6) and determine the abundance, distribution, and phenotypic effects of rare variants in human populations (7, 8).

Population genetic models predict that mutation rates, the strength of selection, and demography affect the abundance of rare variants, although the relative importance of each is a long-standing question (9–11). To understand rare variant diversity in humans, we sequenced 202 genes in a sample of 14,002 well-phenotyped individuals (table S1). These genes represent approximately 1% of the coding genome and approximately 7% of genes considered current or potential drug targets (12) and are enriched for cell-signaling proteins and membrane-bound transporters (table S2). A total of 864 kb were targeted, including 351 kb of coding and 323 kb of untranslated (UTR) exon regions (database S1). More than 93% of target bases were successfully

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sequenced, at a median depth of 27 reads per site (13). Because rare variant discovery can easily be confounded with sequencing errors, we performed numerous experiments to demonstrate high data quality (table S3) (13). The sequenced subjects include two population samples ($n = 1322$ and 2059 subjects) and 12 disease collections ($n = 125$ to 1125 cases) (table S4). The self-reported ancestry of the sample was predominantly European (12,514), African American (594), and South Asian (567). Some of the following analyses focus on the European subset, which is well-powered to investigate rare variants. On the basis of our sample size, we expect that 94% of variant alleles with minor allele frequency (MAF) of 0.01% in Europeans were sampled at least once.

Sequencing revealed an abundance of rare (MAF < 0.5%) single-nucleotide variants (SNVs) compared with common variants (Fig. 1, A and B). We observed on average 1 variant per 17 base

pair (bp) in the overall sample and 1 variant per 21 bp in the Europeans (table S5). Among all variants, more than 95% were rare (MAF ≤ 0.5%), and more than 74% were observed in only one or two subjects. Approximately 90% of rare variants were not previously reported, as opposed to ~5% of common variants (MAF > 0.5%) (fig. S1). For the large European subset, Watterson's θ_W —a metric of genetic diversity (Table 1)—was much larger (40.38×10^{-4}) than in previous smaller-scale studies and an order of magnitude larger than the pairwise metric θ_π (3.96×10^{-4}). We observed a third allele at 2.0% of variable sites, and among those, 1.6% had a fourth allele. We found between 1.2 and 1.9 non-diallelic SNVs per kilobase of sequence (fig. S2), which tended to occur at sites under lower evolutionary conservation (fig. S3) (13). The rate of variant discovery remained nearly constant with increasing sample size (Fig. 2A). We project 111

to 153 variants per kilobase in a sample of 100,000 Europeans and 337 to 452 variants per kilobase in a sample of 1 million (Fig. 2, A and B).

These patterns are at odds with notions that human genetic diversity can be summarized by use of an effective population size (N_e) of 10,000 individuals (14). An N_e of 10,000 individuals is predictive of the average pairwise differences between human sequences (Table 1, θ_π) and is reflective of our emergence from a small population in Africa (15). However, the excess of rare variants observed here ($\theta_W \gg \theta_\pi$) is a signature of the rapid growth and large population sizes that typify more recent human demographic history (8). When we fit a demographic model to the fourfold degenerate synonymous (S) variants in Europeans, we obtained a maximum-likelihood estimate for a recent growth rate of 1.7% [95% confidence interval (CI) = 1.2 to 2.3%] and a recent European effective population size

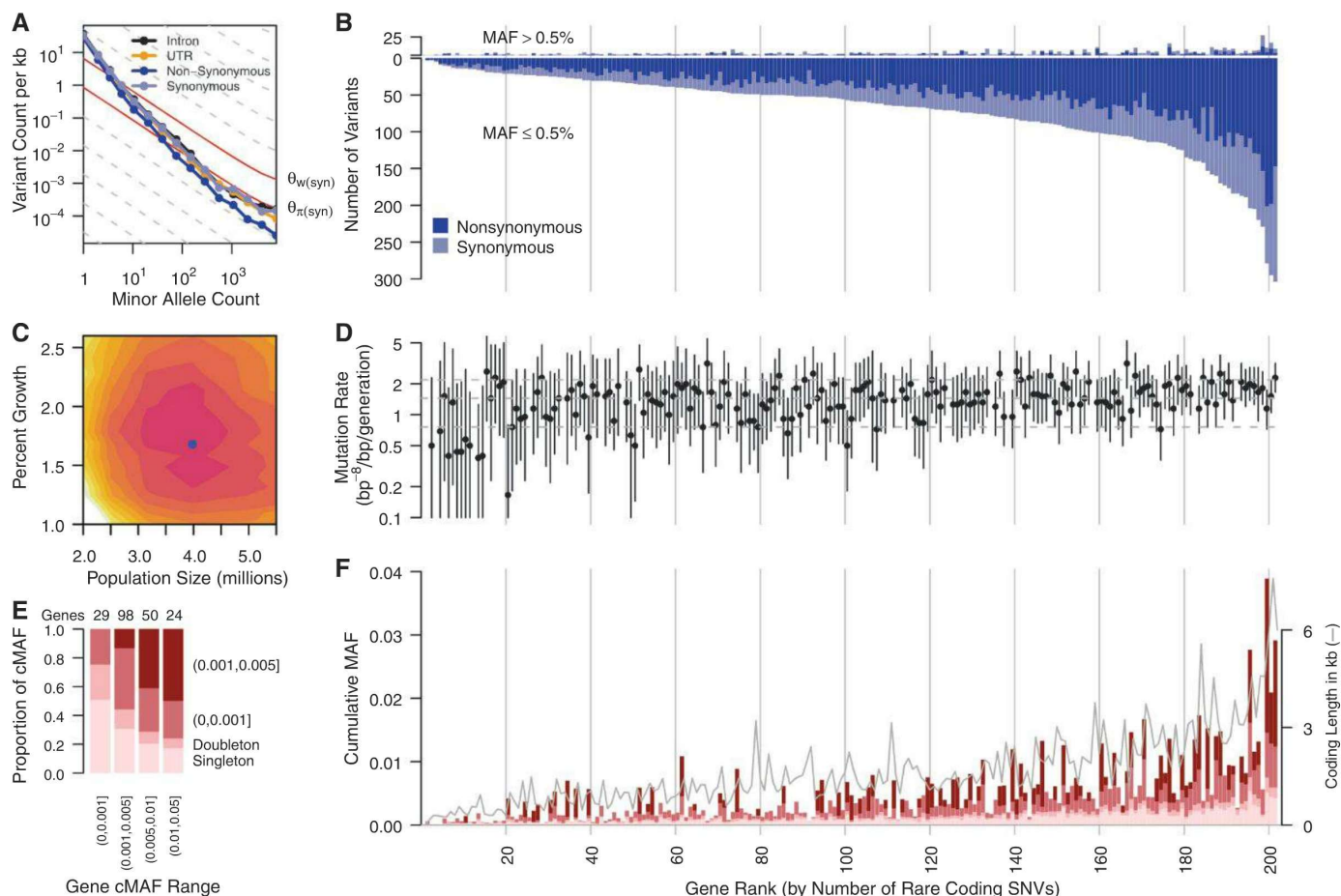


Fig. 1. (A) Frequency spectrum of variants relating the number of variants per kilobase within minor allele counts. Solid red lines provide expectations from nucleotide diversity (θ_π) and the number of segregating sites (θ_W). **(B)** The number of common (MAF > 0.5%, above the origin) and rare (MAF ≤ 0.5%, below the origin) coding variants observed in each gene are shown as stacked bars of NS and S variants. **(C)** Log-likelihood surface of European population growth (r) and population size (N_e) in a demographic model. Colored contours correspond to 2 log-likelihood intervals. The blue point is the maximum likelihood estimate of r and N_e . **(D)** Per-gene mutation rates with 2 log-likelihood intervals. Horizontal lines are 10th, 50th and 90th mutation rate

percentiles. Seven genes on the X chromosome and four genes with low target coverage or yielding too few common variants for inference (*ADRB3*, *CCR5*, *MIF*, and *PTGER1*) were excluded. **(E)** Proportion of rare cMAF accounted for by SNVs of increasing frequency. **(F)** Proportion of rare variants in four cMAF ranges falling within the MAF categories shown in (E). The successfully sequenced coding length of each gene (in kilobase) is overlaid as a gray line. cMAFs in (E) and (F) are for amino acid-changing variants in each gene predicted to be damaging or are evolutionarily conserved (phyloP ≥ 2). Genes in (B), (D), and (F) are ordered by number of rare coding variants per gene, and vertical lines correspond to rank deciles.

of 4.0 million (95% CI = 2.5 million to 5.0 million) (Fig. 1C).

Taking advantage of the large size of this study for population genetics inference (8, 16), we estimated mutation rates for each gene (Fig. 1D) (13) and obtained a median estimate of 1.38×10^{-8} per base pair per generation, with 90% of estimates falling between 1.7×10^{-9} and 2.4×10^{-8} . Incorporating singleton discovery false negative rates from 2 to 8% resulted in median estimates no greater than 1.45×10^{-8} . These population-genetic-based rate estimates are similar to recent pedigree-based mutation rate estimates of 1.36×10^{-8} per base pair per generation (17) and 1.17×10^{-8} per base pair per generation (13, 18). Further, these data reject a model of uniform mutation rates across genes ($P < 2 \times 10^{-8}$) and show synonymous mutation rates are correlated with the number of non-synonymous (NS) rare variants ($P = 0.04$) and guanine-cytosine content ($P < 2.4 \times 10^{-9}$) (13).

The excess of rare variants observed in coding regions is also due to an abundance of NS variants segregating at low frequencies that are not seen at more common variant frequencies as a result of purifying selection. Summing across all frequencies of variant sites, S and intronic variants occurred more frequently (~70 variants per kilobase each) as compared with UTR and NS variant sites (~55 and ~45 per kilobase of UTR or

NS sequence, respectively) (Fig. 2A). Yet, examining the abundance of rare variants across functional categorizations of variant sites reveals little difference among classes when minor allele count is low (Fig. 1A). These patterns are likely due to an equal input of mutations for each category followed by purifying selection preventing deleterious NS and UTR variants from reaching higher frequencies (13, 19). The ratio of NS:S in singletons is close to that expected among new mutations and then decreases with increasing frequency (Fig. 2C). Using the approach of (2), we estimate that although ~70% of all NS sin-

gletons in our sample are sufficiently deleterious that they will never reach frequencies >5%, only 13% of new NS mutations appear so deleterious that they would not be observed even as singletons in a sample of this size (13), putting an upper bound on the frequency of dominant lethal mutations (15). The output of functional prediction algorithms (Fig. 2, D and E) also suggest that rare variants are enriched for damaging variants.

On average, each subject carried a rare minor allele at 0.02% of all NS sites, of which ~56% are expected to be deleterious enough to never be fixed. More than 0.3% of sequenced subjects

Table 1. Comparison of classical population genetic measures of sequence diversity across studies.

Study	Number of genes*	Sample size	Sample†	Length (Mb)	θ_{π} ($\times 10^{-4}$)	θ_w ($\times 10^{-4}$)
Akey (27)	132	23	EU	2.50	3.41	7.35
SeattleSNPs (28)	213	23	EU	7.26	6.81	6.36
Ahituv (29)	58	757	EU	0.13	4.32	10.11
Current study	202	500‡	EU	0.74	3.96	8.79
		11,000	EU	0.74	3.96	40.38
		500‡	SA	0.69	4.04	10.67
Akey <i>et al.</i>	132	24	AA	2.50	4.49	12.10
SeattleSNPs	213	24	AA	7.26	8.97	10.15
Current study	202	500‡	AA	0.70	4.89	13.78

*Studies differ in the relative proportion of coding and noncoding sequences. †Ancestry is indicated as EU, European; AA, African-American; SA, South Asian. ‡Sampled to $n = 500$ subjects.

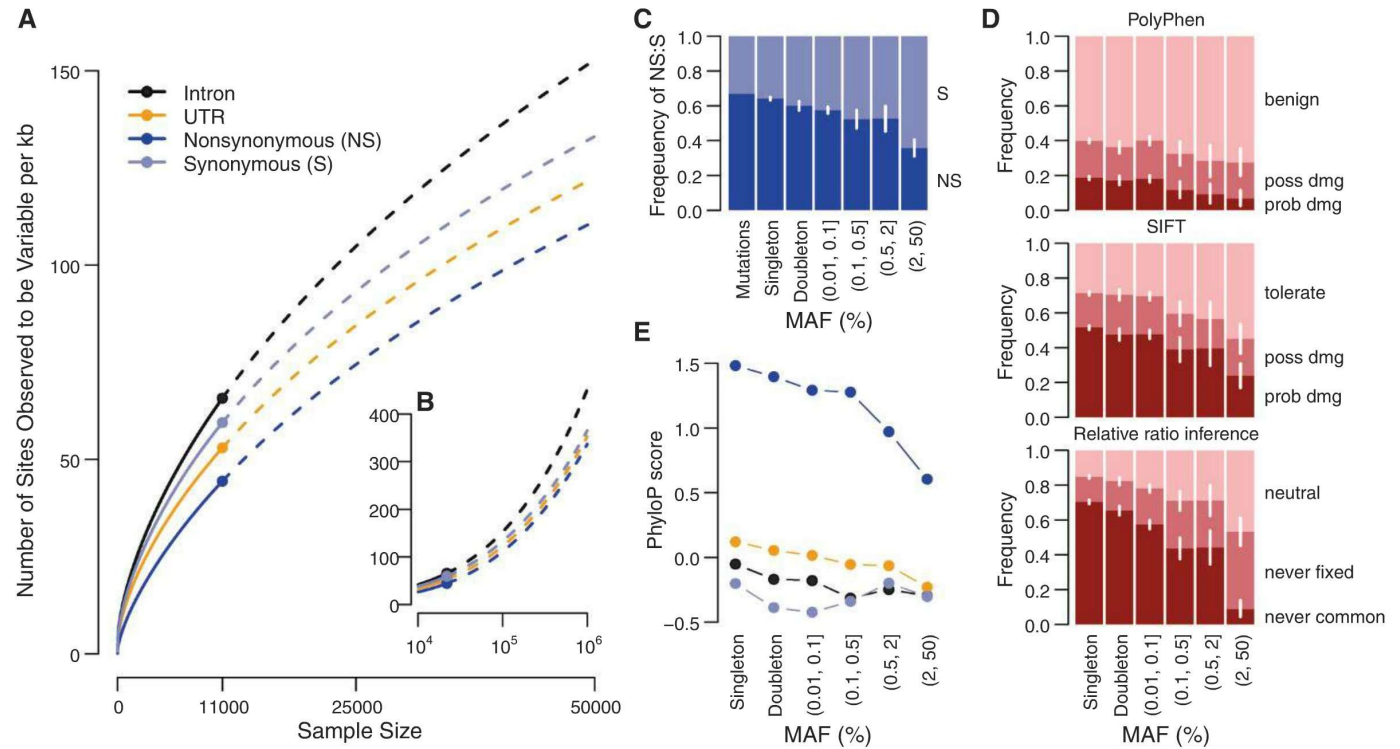


Fig. 2. (A and B) Number of variants per kilobase of intronic, UTR, NS, or S sequence with sample size increasing to 50,000 (A) and 1 million (B) Europeans. Observed numbers are given as a dot, and solid and dashed lines indicate hypergeometric expectations and jackknife projections, respectively. (C) Expected ratios of NS to S variants in the absence of selection and observed ratios for different MAF bins. (D) The proportion of NS variants predicted to be benign,

possibly damaging, or probably damaging by use of PolyPhen or SIFT and the proportion of NS variants that is neutral, deleterious so that they will never become common (MAF > 5%), or never be fixed in Europeans as predicted by the relative ratios of NS:S variant abundances observed at different MAF (2). In (C) and (D), 95% CIs are represented by white lines. (E) phyloP score for intronic, UTR, NS, and S variants for different MAF bins.

carried at least one mutation reported to be a dominant cause of disease (table S6) (13). We also identified variants at $0.5\% < \text{MAF} \leq 2\%$, the so-called goldilocks variants (20), in that they would be common enough to be detected in large population samples and rare enough to be enriched for variants under purifying selection (Fig. 2, C to E). In the European sample, we observed 105 amino acid-changing variants in 73 genes falling within this frequency range. Half of these were predicted to be functionally damaging, relative to 31% of more common coding SNVs ($>2\%$) and 65% of singletons. By comparison, we found 210 goldilocks variants in African Americans and 132 in South Asians, supporting the value of non-European samples for the genetic analysis of complex traits (21).

Rare variants can be tested in aggregate for an association with disease (6), in which the power of the test is strongly correlated with the cumulative MAF (cMAF) of potentially deleterious SNVs within each gene (Fig. 1, E and F, and figs. S4 and S5). Thirty-seven percent of genes had cMAFs $>0.5\%$ of rare alleles predicted to be deleterious. We tested associations of common variants individually and rare coding variants in aggregate with the diseases represented in this study (13). When possible, we matched controls with cases using genome-wide genetic similarity. Nevertheless, type I error rate inflation consistent with effects of population stratification was observed (table S7 and fig. S6) and was worse for rare variant tests. There were no statistically significant rare variant associations and thus no compelling evidence connecting any

genes with the studied diseases. Of 13 more closely examined genes reported to be associated with six of the diseases investigated (table S8) (22), only the association of rare variants in *IL6* with multiple sclerosis was noteworthy ($\text{OR} = 12$, $P = 0.007$) (table S9).

Because rare variants are typically the result of recent mutations, they are expected to be geographically clustered or even private to specific populations. Using a measure of variant sharing between two samples (7), we found that for common variants, any two European populations appear to be panmictic, whereas for rare variants, European populations show lower levels of sharing (fig. S7). In general, the level of sharing depends on geographic distance, with the dependence increasing substantially with decreasing allele frequency (fig. S8). The Finnish population shows substantially lower levels of sharing with other European populations than predicted by geographic distance, which is consistent with hypotheses of a historical Finnish demographic bottleneck (23). Levels of rare variant sharing are even lower when comparing populations from distinct continents. Thus, catalogs of rare variants will need to be generated locally across the globe (7, 24).

We found substantial variation in the total abundance of variants across populations, even within Europe (Fig. 3 and fig. S7D), which is likely due to demographic history. In particular, we observed a north-south gradient in the abundance of rare variants across Europe, with increased numbers of rare variants in Southern Europe and a very small number of variants

among Finns, who had about one third as many variants as southern Europeans. The gradient is consistent with observed gradients in haplotype diversity (25) and a Finnish ancestral bottleneck (23). Association mapping approaches based on rare variant diversity levels will be more susceptible to subtle effects of population stratification (26) and more likely to result in false-positive disease associations.

To evaluate our conclusions relative to the rest of the genome, we compared the NS:S variant ratios of the sequenced genes with the entire coding genome within the low-coverage CEU 1000 Genomes Project data. The average per subject NS:S ratio from our 202 genes was 0.54, whereas all other genes had an average ratio of 0.94 ($P < 10^{-15}$) (fig. S9). By comparison, genes found in Online Mendelian Inheritance in Man (OMIM) and the genome-wide association studies catalog (22) had average ratios of 0.75 and 0.78, respectively. This implies that the genes in this study are under stronger purifying selection, which is consistent with their choice as drug targets and importance to human health. Hence, our results cannot be simply extrapolated to the whole exome. Instead, it is likely that our results underestimate the average genetic diversity that will be found in more typical human gene-coding regions, primarily regarding the amount of NS variation.

This large-scale resequencing study provides a unique description of variation for 202 drug target genes and insight into the very rare spectrum of variation. Although sequencing error might be a concern, we show that the error rates in this study are low (table S3). Another caveat is that our inference of demographic parameters and mutation rates ignores the effects of background selection on synonymous variants. Despite these caveats, the results show there is an abundance of rare variation in human populations and that surveys of common variants are only observing a small fraction of the genetic diversity in any gene. Further, much of the rare variation in coding regions appears to be functional and may be crucial for yielding insights into the genetic basis of human disease. Because the genes studied are related to drug discovery, development, or repositioning efforts, this work has potential to help investigate drug target biology and drug response.

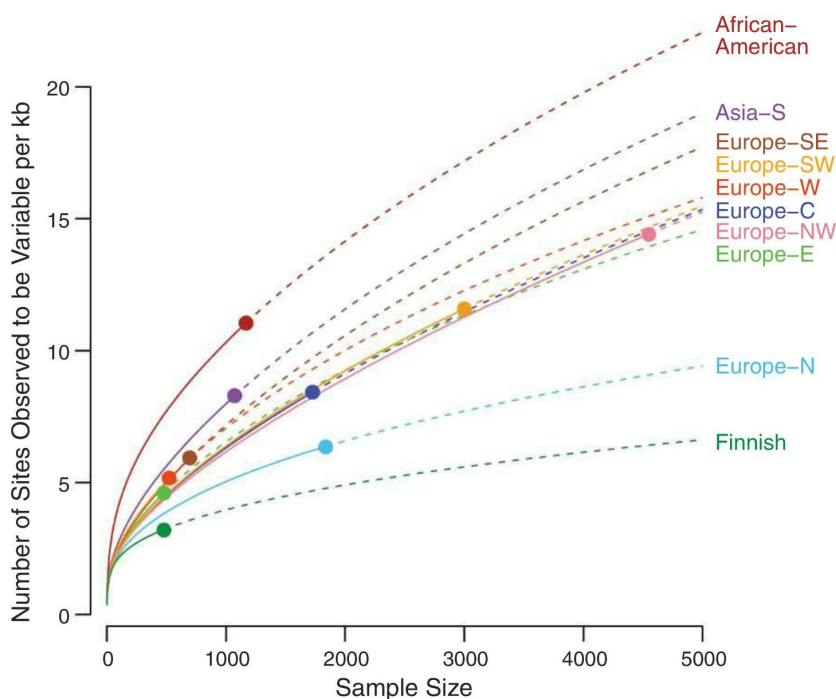


Fig. 3. Number of variants per kilobase of sequence with sample sizes increasing to 5000 people for multiple populations. Observed numbers are given as a dot, and solid and dashed lines indicate hypergeometric expectations and jackknife projections, respectively.

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by a NIH Genome Analysis training grant. All variants described in this study have been submitted to dbSNP; accession nos. are included in database S2. Subject-level sequence data for CoLaus and LOLPOP studies are available in dbGaP. Additional subject-level sequence data can be made available upon request from the authors under a Data Transfer Agreement for the purpose to understand, assess, or extend the conclusions of this paper.

Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1217876/DC1
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Databases S1 to S3

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Recurrent Hemizygous Deletions in Cancers May Optimize Proliferative Potential

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Tumors exhibit numerous recurrent hemizygous focal deletions that contain no known tumor suppressors and are poorly understood. To investigate whether these regions contribute to tumorigenesis, we searched genetically for genes with cancer-relevant properties within these hemizygous deletions. We identified STOP and GO genes, which negatively and positively regulate proliferation, respectively. STOP genes include many known tumor suppressors, whereas GO genes are enriched for essential genes. Analysis of their chromosomal distribution revealed that recurring deletions preferentially overrepresent STOP genes and underrepresent GO genes. We propose a hypothesis called the cancer gene island model, whereby gene islands encompassing high densities of STOP genes and low densities of GO genes are hemizygously deleted to maximize proliferative fitness through cumulative haploinsufficiencies. Because hundreds to thousands of genes are hemizygously deleted per tumor, this mechanism may help to drive tumorigenesis across many cancer types.

Cancer progression is directed by alterations in oncogenes and tumor suppressor genes (TSGs) that provide a competitive advantage to increase proliferation, survival, and metastasis (1–3). The cancer genome is riddled with amplifications, deletions, rearrangements, point mutations, loss of heterozygosity (LOH),

and epigenetic changes that collectively result in tumorigenesis (4–7). How these changes contribute to the disease is a central question in cancer biology. In his “two-hit hypothesis,” Knudson proposed that two mutations in the same gene are required for tumorigenesis, indicating a recessive disease (8). In addition, there are now several examples of haploinsufficient TSGs (9–11). Current models do not explain the recent observation that hemizygous recurrent deletions are found in most tumors (12, 13). Whether multiple genes within such regions contribute to the tumorigenic phenotype remains to be elucidated.

Recent analysis of 3131 tumors revealed 82 regions of recurrent focal deletion (13), averaging six deletions per tumor and 24 genes per deletion (Fig. 1C, fig. S1A, and table S1) (14). Breast, gastric, bladder, pancreatic, and ovarian cancers average ≥10 deletions/tumor (Fig. 1A). Several possible explanations exist for the roles of these

deletions in tumorigenesis. First, they may contain a recessive TSG where mutation or epigenetic silencing of the second allele is necessary for tumorigenesis. Second, they may recur because they mark unstable genomic regions, such as fragile sites (12). Finally, it is possible that single-copy loss may provide a selective advantage irrespective of changes in the remaining allele.

To address the possibility that recurrent deletions are enriched for recessive TSGs, we analyzed these regions for the presence of known or putative recessive TSGs. For this purpose we used a list from the Cancer Gene Census (15) and a list of putative TSGs that we identified with homozygous loss-of-function (termination codon or frameshift) mutations from whole-genome sequencing of 526 tumors in the Catalogue of Somatic Mutations in Cancer (COSMIC) (Fig. 1B and tables S2 and S3) (16). Only 14 of 82 recurrent deletions contained a known TSG, and only 10 had a mutant or putative TSG, 6 of which were in a region with a known TSG (Fig. 1C and fig. S1). Thus, only 18 of 82 deletions can be explained by known or putative recessive TSGs. This number may increase if gene silencing is as prevalent as point mutation for gene inactivation, but this remains to be determined across all cancers. These data suggest that in addition to the two-hit mechanism, an alternative mechanism may function to provide a selective advantage to these deletions.

Of the many altered processes promoting tumorigenesis, proliferation is likely to encompass the most genes, as it is integrated into all developmental decisions. Cancer evolution relies on alterations that provide incremental increases in cell number—a function of cell duplication frequency coupled with cell survival efficiency. The average fitness increase of a single alteration in tumors is estimated to be 0.4% (17). Because subtle changes in proliferation rates can have profound effects on tumor fitness and clonal selection, we examined whether recurrent deletions affect regulators of cell proliferation. We

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define proliferation regulators as falling into two categories: suppressors of tumorigenesis and/or proliferation (STOP genes) that restrain proliferation, and growth enhancers and oncogenes (GO genes) that promote proliferation. By definition, STOP genes contain prominent TSGs that restrain proliferation (e.g., Cdk inhibitors, Rb, and p53), whereas GO genes include essential genes and some that simply enhance proliferation rates. The interplay between STOP and GO genes controls proliferation.

To identify candidate STOP genes, we performed a proliferation screen with a library containing 74,905 short hairpin RNAs (shRNAs) targeting 19,011 genes (18–20) in telomerase-immortalized human mammary epithelial cells (HMECs) (fig. S2A). We chose HMECs because they have intact TSG pathways and should hypothetically be a model for proliferation effects in early tumorigenesis where the neoplasms are less abnormal. By comparing the ratio of each shRNA's abundance (end versus initial sample) after eight population doublings, we identified enriched shRNAs (Fig. 2A, red). Screen data were analyzed as described (20), using significance analysis of microarray (SAM) to identify shRNAs consistently enriched by a factor of 1.8 or more across triplicates [false discovery

rate (FDR) = 5%], representing a $\geq 7.5\%$ increase in cell number per generation. This identified 4496 (6.0%) enriched shRNAs targeting 3582 (18.8%) candidate STOP genes (Fig. 2B and table S4). Of the shRNAs tested, 51% recapitulated in a 5-day multicolor competition assay (MCA) (21) (fig. S2, B and C).

To validate more genes and eliminate off-target effects, we used a large-scale sublibrary validation (Fig. 2C). From 3700 candidate STOP genes from multiple screens (Fig. 2, A and B, fig. S3, A and B, and tables S4 and S5), we chose 1555 genes for validation studies by including only those genes that (i) increased proliferation upon depletion in an independent triplicate rescreen, (ii) were validated by MCA, or (iii) were enriched by a factor of >2 with three or more independent shRNAs. We synthesized a sublibrary against this higher-confidence list with 12 shRNAs per gene and 50 negative control shRNAs targeting firefly luciferase (FF). We performed a secondary validation screen in triplicate and deconvolved samples by Illumina sequencing. Data were normalized for the number of sequencing reads per sample and to the mean of 50 FF shRNAs (table S6) and were analyzed using SAM with FDR = 5%. Sixty percent of the shRNAs increased cell proliferation by a fac-

tor of 2 or more (Fig. 2D and table S7). Many STOP candidates validated with four or more shRNAs enriched by a factor of ≥ 2 (1406 genes), ≥ 4 (878 genes), or ≥ 6 (235 genes) (Fig. 2E). Furthermore, we observed a much larger fraction of shRNAs strongly enriching by a factor of ≥ 4 (30.2%) or ≥ 6 (13.3%) relative to our primary screen. Examination of shRNAs against the known proliferation regulators p53 and Rb revealed that 9 of 13 p53 shRNAs and 9 of 12 Rb shRNAs increased cell proliferation by a factor of 2 or more (fig. S4A). These data indicate that the validation screen can distinguish between authentic regulators of cell proliferation and false positives.

Using a stringent cutoff, analysis of the 878 STOP genes for which four or more shRNAs each resulted in a factor of ≥ 4 increase in cell proliferation revealed many genes involved in cell cycle regulation, apoptosis, and autophagy (Fig. 2F and table S7) and numerous TSGs (fig. S4B). To establish statistical significance for TSG enrichment, we compared our primary and validation gene sets to the list of known TSGs defined by the Cancer Gene Census (Fig. 3A and table S2) (15). This comparison revealed significant enrichment with 44.4% more TSGs than expected in the primary STOP gene set ($P = 0.032$)

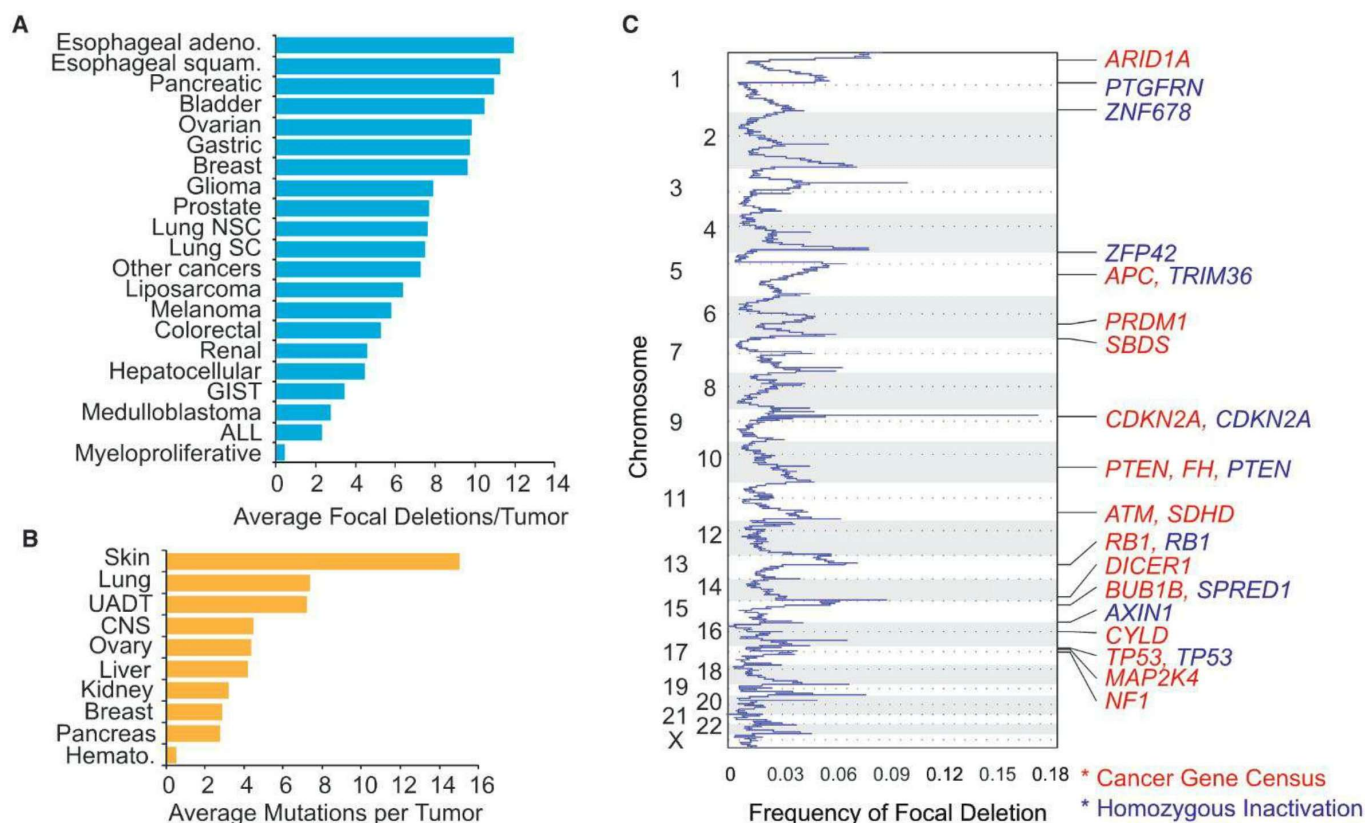


Fig. 1. Most recurrent cancer deletions do not contain known or putative recessive TSGs. (A) Average recurrent focal deletions per tumor for cancer subtypes (adeno., adenocarcinoma; squam., squamous; NSC, non-small cell; SC, small cell; GIST, gastrointestinal stromal tumor; ALL, acute lymphoblastic leukemia). (B) Loss-of-function mutations per tumor from COSMIC, averaged for various cancers (UADT, upper aero-digestive tract; CNS, cen-

tral nervous system; Hemato., hematopoietic). (C) A subset of recurrent cancer deletions contains known or putative tumor suppressors. The frequency of focal deletion was plotted by chromosome location. Red gene names denote the presence of a known TSG from the Cancer Gene Census; blue gene names denote a homozygously inactivated gene from COSMIC whole-genome sequencing.

and 100% more TSGs than expected in the validation screen ($P = 9.1 \times 10^{-3}$). We also compared the STOP candidates to the list of loss-of-function mutations in the 526 tumors in the COSMIC database (Fig. 3B and table S8) (16) and found significant enrichment of primary and validation STOP genes, with 12.9% more primary STOP genes ($P = 1.0 \times 10^{-3}$) and 16.1% more validation STOP genes ($P = 0.019$) exhibiting loss-of-function mutations in cancers. These data indicate that our STOP lists are likely to be enriched for novel TSGs and that genes with loss-of-function mutations found in tumors are enriched for negative regulators of proliferation, arguing that cell proliferation

in this HMEC system is relevant to in vivo tumorigenesis. To examine deletions, we mapped the chromosomal locations of STOP genes relative to recurrent deletions from 3131 tumors (13) (Fig. 3B and table S9). We observed a significant enrichment ($P = 9.1 \times 10^{-4}$) of genes located in recurring deletions in the primary STOP gene set, with 13.6% more genes than expected. The enrichment improved with the validation set to 19.1% more genes than expected ($P = 5.8 \times 10^{-3}$). This enrichment indicates that a significant proportion of the STOP genes identified are likely to functionally restrain tumorigenesis. Additionally, of the 451 observed overlapping genes, to

our knowledge only 6 have been previously implicated as bona fide TSGs (*SMAD4*, *RBI*, *ATM*, *APC*, *PTEN*, and *TP53*), suggesting the existence of many previously undescribed TSGs on this list. The observation that hemizygous recurrent focal deletions contain more STOP genes than expected suggests that multiple genes in each region contribute to tumorigenesis, possibly through haploinsufficiency. The recurrent deletions contain more STOP genes than predicted if only one gene per deletion were contributing to the phenotype. If these deletions preferentially select regions with the highest densities of STOP genes, then one might expect that the STOP gene distribution would

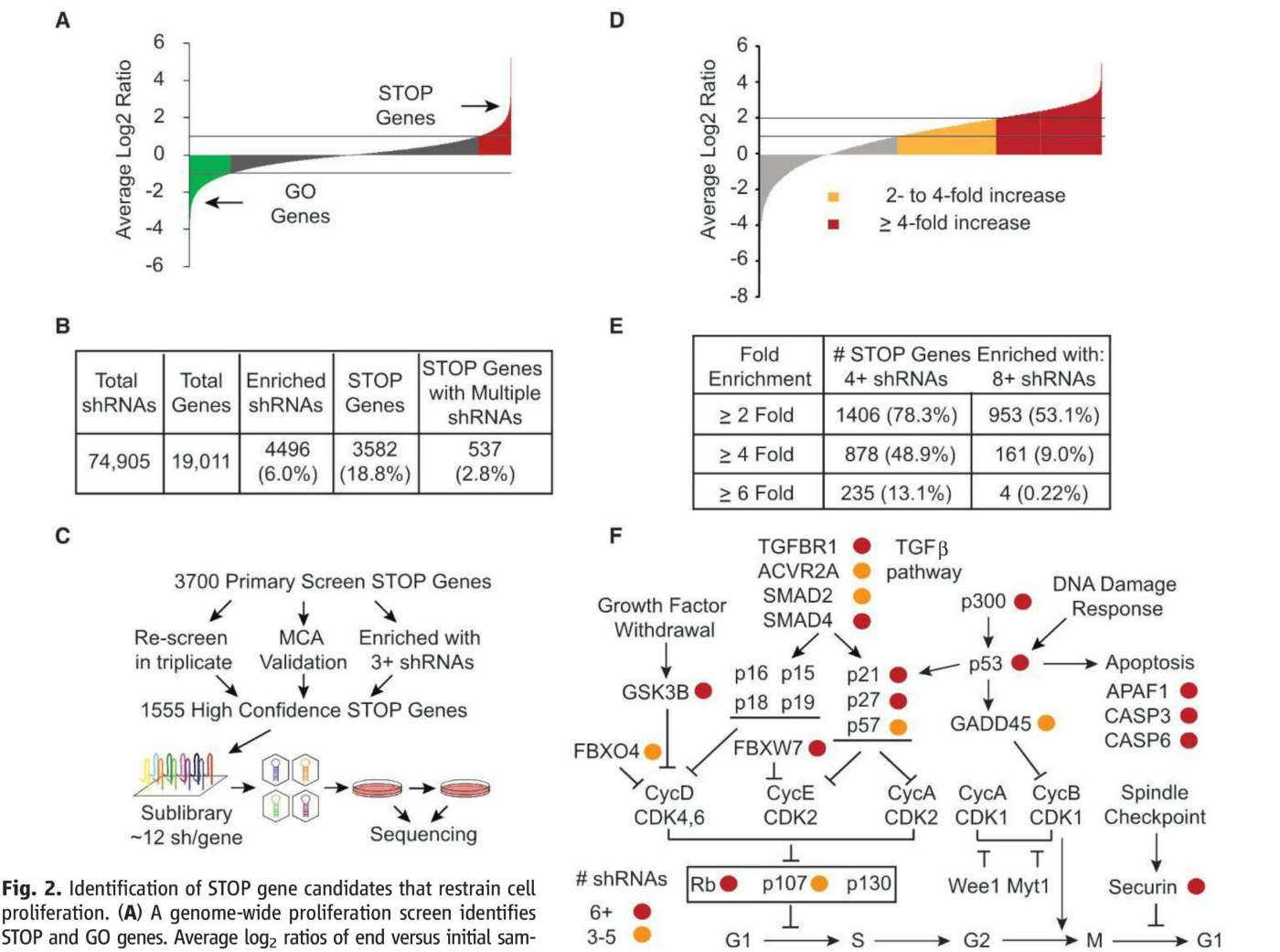


Fig. 2. Identification of STOP gene candidates that restrain cell proliferation. **(A)** A genome-wide proliferation screen identifies STOP and GO genes. Average log₂ ratios of end versus initial samples for >74,000 shRNAs across triplicates were plotted. Enriched shRNAs are denoted as STOP genes (red); lethal shRNAs are denoted as GO genes (green). **(B)** Numbers and percentages of STOP genes identified with single and multiple shRNAs, enriched shRNAs scoring in the primary screen, and total shRNAs and genes screened. **(C)** Generation and screening of a validation sublibrary containing multiple shRNAs per gene. A sublibrary targeting 1555 high-confidence STOP genes was designed containing 12+ additional shRNAs per gene, shRNAs that enriched by a factor of 2 in the primary screens, and control shRNAs. The sublibrary was synthesized using parallel microarray synthesis, cloned, and screened for the ability to increase cell proliferation, using Illumina sequencing for pool deconvolution. **(D)** Average log₂ ratios of end versus initial samples across triplicates for 21,768

shRNAs from the validation screen were normalized for sequencing reads per sample and to the mean of 50 negative control shRNAs targeting FF. shRNAs that increased proliferation (relative to FF controls) by a factor of 2 to 4 are in orange; those that increased proliferation by a factor of 4 or more are in red. **(E)** Validation of STOP genes as assessed by multiple shRNAs. Numbers and percentages of STOP genes with multiple shRNAs are shown, according to increased proliferation by a factor of ≥2, ≥4, or ≥6. **(F)** Known pathways with multiple shRNAs in the validation screen. Genes that validated in the secondary screen by a factor of ≥4 with multiple shRNAs are denoted with circles corresponding to three to five shRNAs (orange) and six or more shRNAs (red).

be significantly different in the recurrent deletions than in other regions of the genome. Thus, we examined the density of STOP gene location within the 82 recurrent deletion peaks relative to the rest of the genome (13). To determine the likelihood of observing the same degree of STOP gene clustering as seen in actual recurrent deletions, we performed a Monte Carlo permutation analysis in which we compared the genes in the original 82 deletion peaks to those generated by random permutation of regions (containing the same number of genes) across a circularized genome. During permutation, the distance between the deletions was fixed to avoid deletion overlaps, and the original deletions were masked from the genome to prevent resampling. We performed 1000 permutations to determine how frequently the same or greater density of STOP genes was observed in randomized deletions, and found that existing cancer deletions specifically encompass regions with high STOP gene density (Fig. 3C and fig. S5A). Significant clustering of STOP genes within recurrent cancer deletions was observed with gene sets from our primary ($P = 5.0 \times 10^{-3}$) and validation screens ($P = 7.0 \times 10^{-3}$). Thus, STOP gene densities equal to that found in recurring deletions were identified only 5 to 7 out of 1000 permutations.

Loss of multiple STOP genes per deletion suggests that cancer cells optimize their proliferative fitness. Increased frequencies of deletions with clusters of STOP genes could occur because the cell now has multiple options for losing the second allele of a recessive TSG or because of

combined haploinsufficiencies. If the latter were a primary driving force by which hemizygous deletions fuel cancer, one would expect that deletions would avoid loss of one copy of essential genes that would limit fitness. To test this, we assembled an in silico list of high-probability essential GO genes involved in critical cellular processes as annotated by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, including DNA, RNA, protein, and fatty acid synthesis (table S10) encompassing 473 genes. This set demonstrated a significant depletion ($P = 0.012$) from recurrent deletion regions, with 28.3% fewer genes present in deletion regions than expected (Fig. 4A). This in silico analysis suggests that the loss of a single copy of GO genes has a negative impact on cellular fitness.

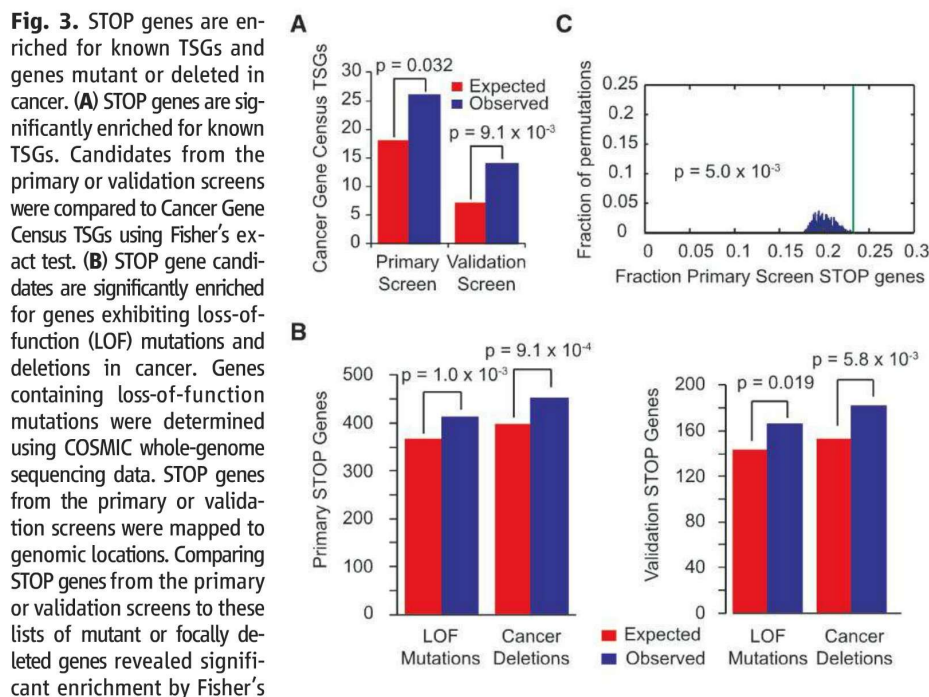
To independently test this hypothesis, we turned to the other arm of our screen that identified candidate GO genes whose depletion limits proliferation and survival. Because both normal and cancer cells are dependent on these essential GO genes, we analyzed data from proliferation screens on HMECs, one normal prostate epithelial cell line, and seven breast or prostate cancer cell lines for shRNAs that reduced cell proliferation and viability by a factor of ≥ 1.5 in five of the nine cell lines (table S11). This GO gene set is enriched for essential core cellular machinery such as the ribosome, spliceosome, RNA polymerase, and DNA replication required for proliferation (Fig. 4B). We observed a significant depletion ($P = 7.6 \times 10^{-3}$) of GO genes located in recurrent deletions, with 22.1% fewer GO genes

than expected (Fig. 4C). When we examined the location of GO genes within recurrent deletions, we found that more than half (58.5%) of deletion regions contained zero GO genes. In contrast to STOP genes, Monte Carlo permutation analysis confirmed that recurrent deletions exist in regions with unusually low GO gene density ($P = 0.011$) (Fig. 4D).

A potential caveat to the interpretation that GO gene depletion reflects haploinsufficiency is that a substantial fraction of recurrent deletions might actually be homozygous. However, our examination of 611 cell lines used in the recurrent deletion analysis (13) revealed that only 5.4% of all genes were ever homozygously deleted, similar to the 11% reported previously (12). Fewer than 1% of genes within deletion regions were homozygous, which suggests that the majority of focal deletions are hemizygous. This low level of homozygous deletion cannot account for the 22 to 28% depletion of GO genes observed, indicating that the absence is more likely due to haploinsufficiency of hemizygous deletions. If such frequent haploinsufficiency occurs among GO genes, by analogy, it is likely that other genes such as the STOP genes also display a similar frequency of haploinsufficiency; if so, this would imply that haploinsufficiency of both STOP and GO genes in sporadic tumors drives tumorigenesis. One possible explanation for this higher than expected frequency of haploinsufficiency is monoallelic expression, a phenomenon in which there is an imbalance in expression levels from the two alleles of a given gene. This imbalance may occur in up to 10% of genes (22). Deletion of the higher-expressing allele could produce a more penetrant haploinsufficient phenotype.

Our analysis found that only 22% of recurrent deletion regions could potentially be explained by known or putative recessive TSGs (Fig. 1C and fig. S1). If most recurrent deletions primarily represent passenger alterations caused by location in a deletion-prone region such as a fragile site, the genes in these regions should possess no special properties. However, we find the opposite to be true, namely that STOP and GO genes exhibit significantly skewed distributions in regions frequently deleted across cancers. Thus, an additional mechanism of cancer evolution may exist that involves selection of hemizygous somatic deletions encompassing high densities of STOP genes and low densities of GO genes. This strategy promotes net proliferation and survival due to the cumulative reduction in dosage of genes with tumor-suppressive properties while avoiding deleterious effects due to reduced dosage of genes that promote proliferation.

Our analysis suggests that ~20% of human genes might display haploinsufficiency, which could have important implications for human health and development given the wide copy number variation seen in humans. Supporting this hypothesis of widespread haploinsufficiency, a number of genes thought to be classical two-hit tumor suppressors also display haploinsufficiency



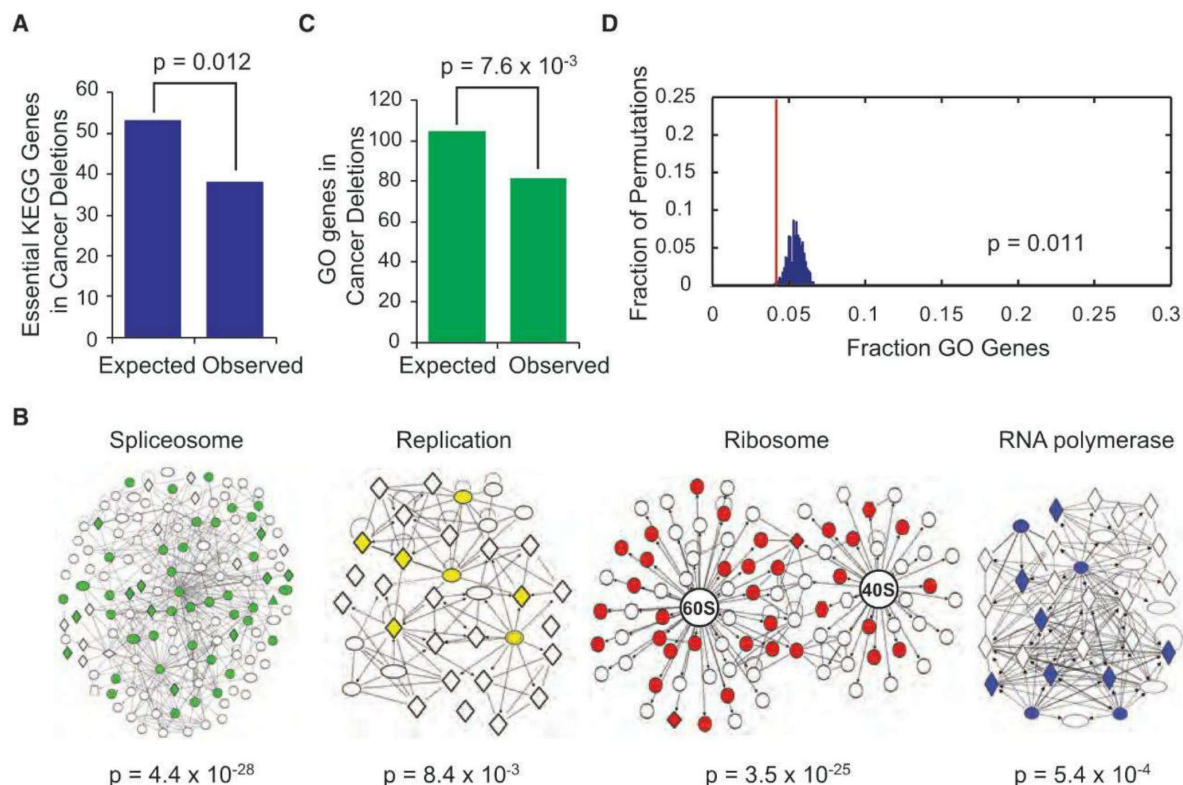


Fig. 4. Hemizygous focal deletions avoid essential GO genes. **(A)** Essential KEGG genes are depleted from cancer deletion peak regions. An essential KEGG gene set representing essential GO genes was assembled using all genes from the following processes: basal transcription and RNA polymerase, the spliceosome, the ribosome, DNA replication, fatty acid biosynthesis, amino-acyl tRNA synthesis, and mRNA transport. Genes were mapped to chromosomal locations and compared to genes found in recurrent cancer deletions. Significant depletion from recurrent deletions was observed using Fisher's exact test. **(B)** GO genes are enriched for genes involved in transcription, splicing, translation, and DNA replication. All genes included in the ribosome, spliceosome, RNA polymerase, and DNA replication KEGG pathways were assembled into interaction modules by means of Ingenuity

Pathway Analysis (Ingenuity Systems, Redwood City, California). GO genes are colored red, green, blue, and yellow, respectively, within each module to demonstrate enrichment for these pathways within the GO gene set using Fisher's exact test. **(C)** GO gene density is lower than expected in cancer deletions. GO genes were mapped to genomic locations and compared to genes found in deletions. Significant depletion was observed between GO genes and genes found in cancer deletion peaks using Fisher's exact test. **(D)** GO genes are significantly depleted from cancer deletions. The GO gene set was mapped to genomic location, and cancer deletion peak regions were overlaid. The percentage of GO genes found in cancer deletions (red line) was compared to the distribution observed after 1000 permutations of the deletion peak regions across the genome.

(9, 23, 24). To provide a simple way to discuss this hypothetical cumulative mechanism, we refer to it as the "cancer gene island model." This model is consistent with the theory of clonal evolution because these deletions provide a selective value to the cell by allowing them to clonally expand, unlike a truly recessive TSG mutation.

Our study provides experimental and statistical evidence that large hemizygous deletions containing islands of clustered proliferation-inhibitory genes are preferentially selected during tumorigenesis, indicating that cancers may exhibit properties of a contiguous gene syndrome. Partial gene dosage due to deletion of multiple adjacent genes in a single deletion region is known to cause several classical contiguous gene syndromes, such as 22q11.2 deletion syndrome. Although we have analyzed proliferation and survival genes, cancer-relevant haploinsufficient genes affecting other aspects of tumorigenesis may also exist in these deletion regions.

If a halving of gene dosage can cause a phenotype, then subtle increases in gene dosage

may also. In addition to deletions, recurrent amplifications are also found in cancers (13). If observations consistent with the cancer gene island model can be extended to gain-of-function mutations, then amplification regions may show enrichment for GO genes whose overproduction enhances proliferation. Recent functional analyses of gene amplifications in hepatocellular carcinoma (HCC) revealed that adjacent genes in the 11q13.3 amplicon (*CCND1* and *FGF19*) and the 11q22 amplicon (*BIRC2* and *YAP1*) are cancer-driving oncogenes in HCC (25, 26). Thus, some amplifications in cancer may also represent contiguous gene syndromes.

The enrichment for genes localized to deletions suggests that we have identified dozens of new TSGs in recurrent deletions. We have also likely identified more TSGs outside of these regions because the STOP gene set is (i) enriched for known TSGs, many of which are not found in recurrent deletions, and (ii) enriched for genes that undergo somatic loss-of-function mutation. Finally, this work suggests that cells possess a

substantial number of genes that restrain proliferation in vitro, which could be inactivated to promote clonal expansion during tumorigenesis in addition to the traditional driver genes currently known.

Given the prevalence of multiple, large, recurring hemizygous deletions encompassing skewed distributions of growth control genes in tumors, we propose that the elimination of cancer gene islands that optimize fitness through cumulative haploinsufficiencies may play an important role in driving tumorigenesis, with implications for the way in which we think about cancer evolution.

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Supplementary Materials

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Materials and Methods

Supplementary Text

Figs. S1 to S6

Tables S1 to S12

References (27–32)

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A Distinct Role of the Temporal-Parietal Junction in Predicting Socially Guided Decisions

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To make adaptive decisions in a social context, humans must identify relevant agents in the environment, infer their underlying strategies and motivations, and predict their upcoming actions. We used functional magnetic resonance imaging, in conjunction with combinatorial multivariate pattern analysis, to predict human participants' subsequent decisions in an incentive-compatible poker game. We found that signals from the temporal-parietal junction provided unique information about the nature of the upcoming decision, and that information was specific to decisions against agents who were both social and relevant for future behavior.

By observing the choices and behavioral cues of others, individuals can learn about, and rapidly adapt their behavior to, dynamic environments (1, 2). Indeed, social information obtained by observing other agents' actions optimizes the learning of environmental contingencies (3). Mental state attribution is the lynchpin in this process, enabling individuals to decipher the motivations underlying the behavior of other agents and predict their upcoming actions (1, 4, 5). Different agents have different relevance for behavior, and failing to distinguish relevant from irrelevant agents may lead to following deceptive advice (6) or conforming with the attitudes or opinions of others (7, 8). Accordingly, some of the most striking examples of human social cognition reflect the differentiation between more and less important social partners: more heavily weighting the preferences of key players when making group decisions (9, 10), discriminating strategic actions from random behavior (11), and comparing oneself to more similar others (12).

A key challenge for neural models of social cognition comes from the very ubiquity of social signals. Social signals in the natural environment are frequent, highly salient, and good predictors of needed future behavior. Information derived from social agents, therefore, might be processed by generalized neural systems that are geared toward identifying any important events in the environment rather than by specialized systems that carry information in specifically social contexts. This challenge and the difficulty in distinguishing its predictions from that of social cognition models lie at the heart of debates in the field (13, 14).

To explore the interaction of social agency with behavioral relevance, we used a simplified poker game (15) in which participants ($n = 18$) played against human and computer opponents (Fig. 1). The opponents alternated across eight functional runs, and the same real human opponent competed against all participants in an independent and incentive-compatible manner (Methods). The human and computer opponents were matched on overall decision probabilities so that differences in observed behavior and brain function could be attributed to perceived social agency.

In each trial, participants first viewed a picture of their opponent. Participants were then presented with either a high card that would win

if they chose to bet and were called or a low card, indicating that would lose if they chose to bet and were called. When the participant did bet, control of the game passed to the opponent who then decided to call or fold (i.e., bet by adding more money or fold by ceding the pot to the participant). Participants won money in this game when opponents bet on trials when they held high cards or folded on trials where the participant bet while holding a low card. Thus, participants maximized earnings in this game through bluffs that prevented the opponent from accurately guessing the card that was held. On average, participants bluffed 54% of the time (range from 29 to 73%), consistent with the equilibrium solution to this game (15). Participants' bluffing decisions were significantly influenced by their opponent's behavior on the previous trial [$t(17) = 8.8$, $P < 0.001$], with greater effects found for human compared with computer opponents [$t(17) = 2.3$, $P = 0.032$].

Our functional magnetic resonance imaging (fMRI) analyses used multivariate pattern analysis (MVPA) at the time of card presentation to predict the participant's subsequent decision, which occurred 6 s later in the trial. We conducted a combinatorial whole-brain analysis that allowed estimation of the unique information carried within local brain segments (16, 17). For every segment, we calculated the average increase or decrease in predictive performance when paired with other brain segments (Materials). We refer to this quantity as the unique combinatorial performance (UCP). A high UCP means that a region carries information that can predict future behavior beyond that obtained from other brain regions. A low UCP means that information within a region is largely redundant with that of other regions. Across both opponents, significant UCP was found in regions often associated with social cognition, including regions in both the dorsal and the medial prefrontal cortices (table S2).

A standard approach for establishing functional specificity in neuroscience research is subtraction, that is, statistical comparison of two conditions that differ only in one process of interest. However, activations identified by subtraction may not be unique to the process of interest

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(Fig. 2C). In contexts like the current task, signals associated with selective attention and vigilance may be present in both social and nonsocial contexts but are more engaged when a social stimulus is present (18, 19). To remove these general cognitive effects, we regressed UCP for a human opponent against UCP for a computer opponent (Fig. 2B). Across regions, there was a robust correlation with a slope greater than one (slope = 1.4, $P = 0.016$). The general consistency of this

response—including for brain regions often described as constituting a social network—implies that the processes engaged during the performance of this task are greater for, but not specific to, social context.

To quantify whether any local brain regions carried distinctly social information, we defined a metric of social bias (Fig. 2D) by using the normalized residual distance from the whole-brain regression line shown in Fig. 2B (Methods). A

region with a positive social bias has a greater UCP against the human opponent than against the computer opponent. One region, the temporal-parietal junction (TPJ), exhibited an extreme social bias that was five standard deviations higher than the mean of all other brain segments (Fig. 2E). The gap between the social bias found in the TPJ and that found in the second-most-biased region was greater than the range of variation throughout the remainder of the brain. This in-

Fig. 1. A single-card poker game. Each trial begins with a picture of the opponent (i.e., a photograph of the human opponent or a computer). The participant's card is revealed, and they then decide whether to bet (B) or fold (F). Buttons then appear, with left/right position randomized across trials, and the participant indicates his or her response. If the participant chooses to fold, the trial ends. If the participant chooses to bet on the card, control passes to the opponent, who has 3 s to decide whether or not to match the participant's bet or fold. Predictions are made by using fMRI data associated with the time period highlighted in green.

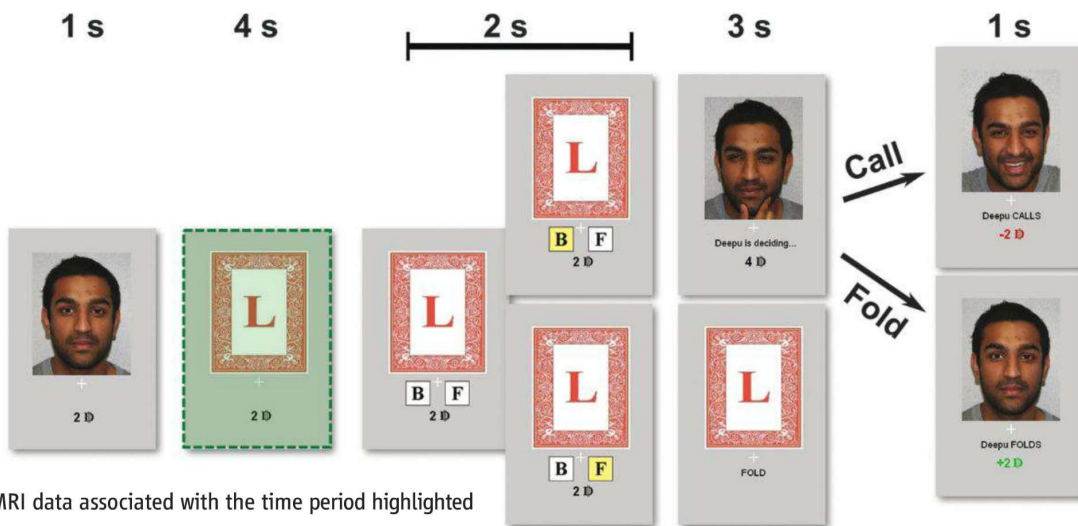
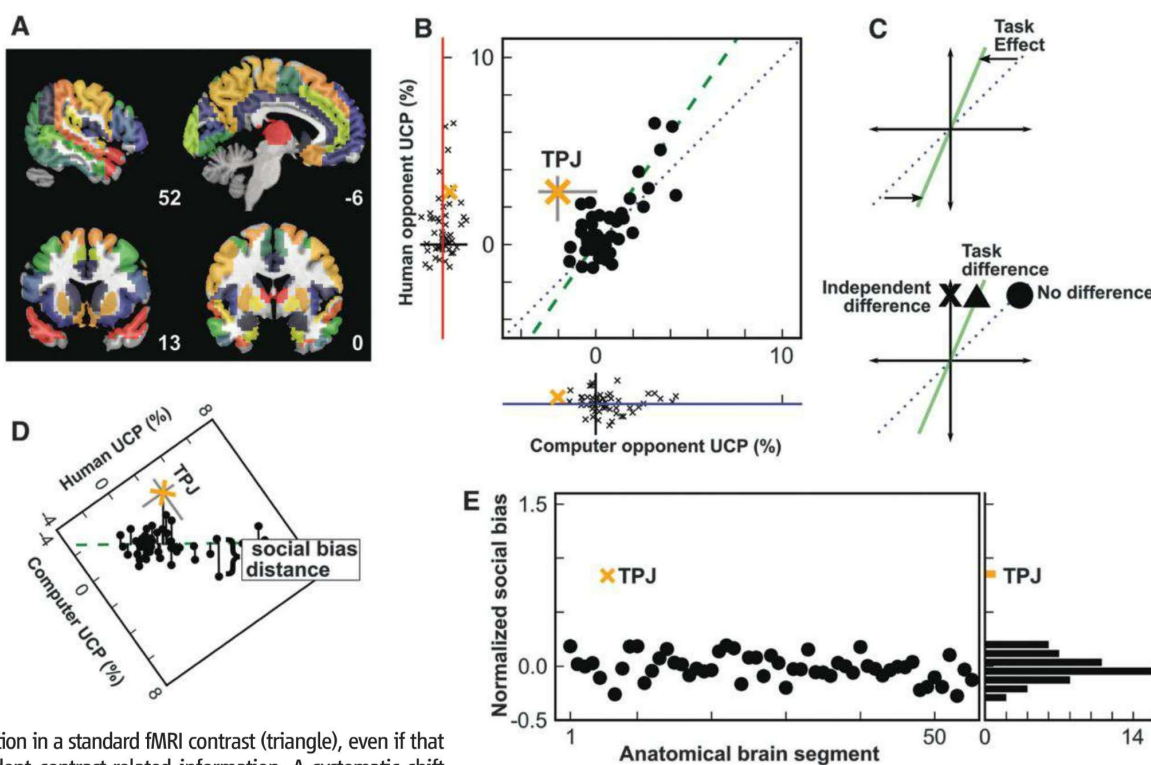


Fig. 2. TPJ plays a distinct role in predicting social decisions. (A) Whole-brain fMRI data was segmented into 55 bilateral anatomical regions, as described in Materials. (B) UCP for a human opponent was regressed against UCP for a computer opponent, across regions (green dashed line, slope = 1.4). For the range reported here, negative values of UCP correspond to a return to chance levels and not negatively predictive models. (C) The slope of the regression line in (B) is consistent with a general task effect (top), such that social contexts tend to engage task-related regions across the brain more than similar nonsocial contexts. A region that was highly engaged by the task might exhibit activation in a standard fMRI contrast (triangle), even if that region carried no independent contrast-related information. A systematic shift away from the regression line (x) would be necessary to show an independent effect in a region. (D) Social bias was defined by the residual difference (social minus nonsocial) in predictive power from the whole-brain regression line. (E) The magnitude of social bias in each region (normalized by the variance estimates for



each segment's UCP), sorted by UCP against the human opponent. Only one region, the TPJ, contributed significantly more UCP against a human opponent than against a computer opponent, independent of the overall task effect.

dependent contribution of the TPJ was robust to multiple forms of correction for brain segment characteristics (figs. S5 and S6) and to Bonferroni correction for multiple comparisons (Materials).

These findings identify a unique and independent role for the TPJ in representing information predictive of behavioral actions during social interactions. To rule out any potential biasing effects of this particular segmentation approach, which defined segments of the brain according to prior anatomical and functional divisions, we repeated all analyses by using random, voxel-based segmentations of the brain (Materials). Voxel-based UCP metrics were then calculated by using the median UCP across the different segmentations (fig. S7). The analysis replicated both previous findings: a nonspecific overall increase in UCP against human compared with computer opponents (slope of 1.4, across all gray matter voxels) but strong social bias in the TPJ (fig. S7D). Moreover, the specificity of TPJ was maintained when extending analyses to combinations of three regions (figs. S9 and S10).

We next evaluated how the subjective behavioral relevance of each opponent modulated information within the TPJ. After completion of the scanning session, participants indicated whether the human or the computer was a better opponent. The social bias found in TPJ was present only for participants who judged the human opponent to be superior (Fig. 3A); again, its bias was more than five standard deviations from the mean normalized residual (Fig. 3B). Conversely, no social bias was observed in TPJ for those who judged the computer opponent as superior to the human [$t(6) = 1.3$, $P = 0.26$; difference between groups: $t(16) = 2.6$, $P = 0.02$].

For comparison, we also plotted UCP for the medial prefrontal cortex (MPFC) and medial posterior cortex (MPC), a network of anatomical regions that, along with the TPJ, have been linked

to aspects of social cognition (20–23). None of these regions exhibited a significant social bias. These results were robust to neural segmentation method selection, with analyses using random voxel-based neural segmentation producing very similar results (fig. S8). Our findings indicate the MPFC and MPC contribute similarly to predicting future behavior in both our social and nonsocial settings, suggesting that they support computations that are critical for social cognition but also applied in at least some nonsocial settings (24, 25).

Our results point to a specific role for TPJ within the social cognition network and demonstrate the unique sensitivity of this region to perceived behavioral relevance of other agents. The TPJ stands out as a contributor of unique and independent social information, even compared with other regions considered to be recruited by social cognition (Figs. 2 and 3). This finding indicates a potential reconciliation for the opposing perspectives that have been proffered for TPJ function: mental inference (26) and orienting to salient external stimuli (14, 27). Responses in the TPJ during social cognition tasks may reflect the nexus of these two operations—that is, interacting with an opponent whose internal states can be modeled (i.e., another human) and whose behavior is also relevant for guiding one's future actions. This interpretation is consistent with demonstrations that TPJ is activated when inferring an adviser's motives when the advice is relevant for an upcoming decision (6) or on potentially highly rewarding trials when implementing an advanced strategy during an interactive choice game (28). However, the present results demonstrate that the TPJ is not generally recruited by external social stimuli but specifically contributes to choice deliberation when modeling the behavioral and cognitive tendencies of a social agent. Accordingly, when others are of lower status (29) or not considered relevant for future behavior,

as in the case of dehumanization (30), the TPJ may be disengaged. The sensitivity of TPJ to both social context and perceived relevance highlights a critical role for this region in coordinating behavior in a dynamic, social environment and demonstrates the capacity of behavioral relevance to modify neural signals.

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Supplementary Materials

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Materials and Methods

Supplementary Text

Figs. S1 to S11

Tables S1 to S5

References (31–60)

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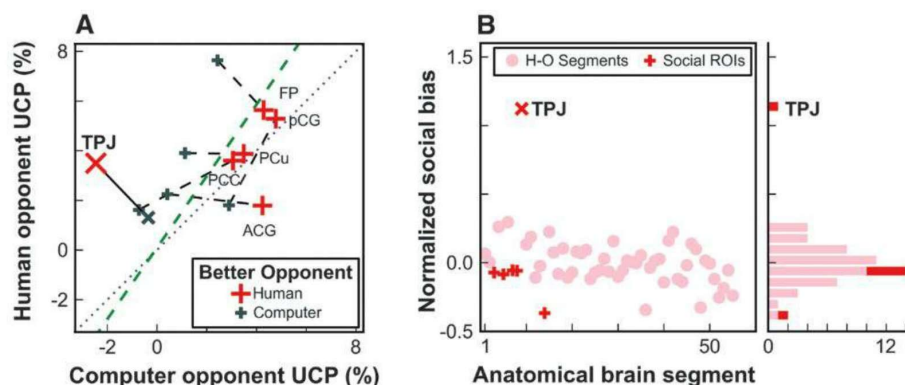


Fig. 3. Social bias in the TPJ depends on the participant's evaluation of the opponent. (A) The social bias observed in the TPJ was found only for those participants who considered the human to be a more informative opponent. Other putative social cognition regions do not show the same relationship with behavioral relevance (regions constituting MPFC: FP indicates frontal pole; pCG, paracingulate gyrus, and ACG, anterior cingulate gyrus; regions constituting MPC: PCC is posterior cingulate cortex and PCu, precuneus). (B) Social bias in all regions of interest (ROI); normalized by the variance estimates for each segment's UCP, sorted by UCP against the human opponent.

Nanotechnology

Prognosis for Nanotechnology Treatments

Nanotechnology-based tools and treatments promise sensitive disease diagnosis and accurate drug delivery. Most important, the physical features of nanometer-size materials provide capabilities that larger objects cannot match. Nonetheless, some of the challenges, such as biocompatibility and toxicity, require ongoing research and improved solutions. Despite these obstacles, some nanoscale approaches to medicine can already be used. **By Mike May**

"The size depends on the application, but targeting specific delivery sites usually requires a small particle to get it there."

Around the world, biomedical researchers are exploring the possible ways that nanotechnology could enhance human health. At the **University of Melbourne** in Australia, for example, Frank Caruso, professor of chemical and biomolecular engineering, explores a variety of self-assembly strategies to create particles for potential use as therapeutics. "We develop systems with defined physical and chemical properties to nanoengineer these systems to enhance payload delivery and achieve site specificity," says Caruso. For drug delivery, the particles can be in the range of roughly 20 nm to 1 μ m. "The size depends on the application," Caruso says, "but targeting specific delivery sites usually requires a small particle to get it there." He adds, though, that the actual size constraints also depend on a particle's shape, plus mechanical properties like elasticity and even the particle's surface chemistry.

The ideal drug delivery system should possess several features. Of course, it needs to get the drug to the intended target, but there's more. The particle must also be biocompatible and biodegradable, so that the cellular machinery can break down the particle to release the therapeutic. Then, the remaining particle components need to be nontoxic. It seems like a lot to ask, but Caruso says, "There's an enormous range of polymers that can be used for assembling particles."

For example, Caruso and his team developed a polymer-based carrier with a roughly 10 nm-thick wall that could transport drugs. "It provides highly elastic mechanical properties that can be tuned," Caruso says. "We're working on understanding how size, shape, and elasticity influence biological interactions, and this helps us nanoengineer the control of drug release and the particle's circulation lifetime." He makes sure to add that such work depends on a multidisciplinary team, including biologists, chemists, immunologists, materials scientists, medical researchers, and more.

EASIER NANO-VIEWS

Some medical applications could benefit from a nanoscale view, like the one provided by field emission scanning electron microscopy (FESEM). Compared with conventional SEM, says Craig Schwandt, senior research scientist at **McCrone Associates** in Westmont, Illinois, "The biggest difference is the diameter of the beam, which is 100 to 1,000 times narrower in FESEM." As a result, FESEM can distinguish structures that lie closer together than SEM can. "The best resolution for SEM is usually about 500 nm apart," says Schwandt, "but FESEM can resolve artifacts that are only 1 nm apart."

Robert Karlinsey, founder of **Indiana Nanotech** in Indianapolis, approached McCrone Associates about applying FESEM to a nano-size dental challenge. Karlinsey's company developed a toothpaste called Clinpro 5000 (sold through a partnership with 3M in St. Paul, Minnesota) that reduces tooth sensitivity to pressure and temperature. Karlinsey wanted to understand the underlying cause of this improvement. Typically, tooth sensitivity arises from nerves inside the dentin. Karlinsey wondered if the toothpaste could be blocking the tubules that provide a pathway to the nerves, so that's where he wanted to look.

"The dentin tubules are just over a micrometer in cross-section," says Schwandt. "Conventional SEM can't see down into the tubules." However, FESEM images were able to reveal spheres—ranging in size from 100–500 nm in diameter—within the tubules after the toothpaste was applied.

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This is just one example of a FESEM application in the realm of nano-imaging and medicine. As Schwandt says, “FESEM can almost get to the resolution of transmission electron microscopy.” He adds, though, that transmission electron microscopy requires extremely thin samples and other modifications that raise concerns over introducing artifacts. With FESEM, a sample requires virtually no preparation, which increases one’s confidence in generating accurate images.

Nano-size particles can also be used to monitor physiological processes. As an example, **Philips Research** in Hamburg, Germany, uses nanotechnology with magnetic particle imaging (MPI). In this technology, roughly 20 nm in diameter particles of iron-oxide get injected into the bloodstream and a specially designed magnetic field causes the particles to orient, like millions of compass needles all aligned along field lines, except for in a small area called the field-free point, where the field is zero. Then, applying an alternating electromagnetic field point causes the nanoparticles in the field-free point to oscillate. An antenna measures this oscillation, which correlates to the nanoparticle-concentration in the field-free point. The Philips platform will scan the field-free point over any desired area.

Jörn Borgert, a Philips senior scientist and project manager, says, “You could use MPI to measure the volume of blood ejected by the heart or measure the amount of blood in any location.”

In the future, the technique may be used in cardiovascular diagnostics. “MPI has the potential to be several hundred times more sensitive than MRI in detecting the nanoparticles,” says Borgert. “This may, in one examination, provide a physician with a comprehensive overview of the cardiac system by tracking nanoparticles through the body.”

So far, this product is not for use with human patients and is still in the research phase. “We’ve demonstrated feasibility in a technical sense, and we’re building a big enough system to find out if it works in a whole-body scenario,” says Borgert.

VIRAL SURVEILLANCE

In the United States, sepsis—a life-threatening condition that can arise from an infection caused by bacteria, fungi, or viruses—impacts almost two million people each year. A nanotechnology-based tool from **Nanosphere** in Northbrook, Illinois, however, helps medical professionals detect this infection from a blood sample and determine which drugs will be most effective.

The nanotechnology lies inside this system. “Verigene uses 13 nm in diameter gold particles with oligonucleotides stuck to their surface,” says William Moffitt, Nanosphere’s chief executive officer and president. For the blood stream–infection panel, oligonucleotides add function to the surface of the Verigene



“Pretty much any disease is targetable. It will be a very general approach.”

particles, which then bind to nucleic acids from the infection-causing pathogens. “Gold nanoparticles with oligonucleotides make highly selective probes,” says Moffitt. “They are very specific to the targets they will bind to, which makes the assays extremely accurate.”

Current methods for diagnosing sepsis can take up to 72 hours or even longer, but the Verigene platform provides results in about two hours. The Verigene assay starts with a blood sample

that goes into the Verigene Reader, which includes a cartridge for the sepsis assays. Roger Moody, Nanosphere’s chief financial officer, calls the reader an “electromechanical device that manipulates a self-contained cartridge, which contains the reagents needed to perform a test.” Nanosphere’s sepsis assay remains under review by the U.S. Food and Drug Administration.

The company has a number of other assays in development. For example, Nanosphere has a test in clinical trials for *Clostridium difficile*, a bacterial infection that is growing in prevalence and can be life threatening. It also makes an influenza assay cartridge that is already approved for clinical use in the United States. Some other tests currently under way include ones for various forms of bacterial infections, intestinal infections, and genetic diseases.

CHIP TECHNOLOGY FOR TREATMENTS

As an expert in polymer chemistry at **IBM** for a quarter century, Jim Hedrick understands how to develop computer-chip applications to, as he says, “make computers perform at outrageous speeds.” That requires plenty of nanotech-knowhow. When he met Yi Yan Yang, an expert in nanomedicine at the **Institute of Bioengineering and Nanotechnology** in Singapore, they discussed how microelectronics might impact medicine.

As a start, Hedrick and Yang worked on developing nanotechnology-based antimicrobials to make the most of their capabilities. “With Jim’s chemical expertise,” Yang says, “we can make polymers with predictable molecular weights, narrow molecular weight distributions, and controlled end-groups, which is an important feature for therapeutic polymers because different molecular weights provide a different pharmacological activity in the body.” Furthermore, the nano-size antimicrobial polymer assemblies possess a slight positive charge that attracts these polymers to the slightly negative bacterial cells. “So you can target these microbes,” Hedrick says. Upon contacting a bacterial cell, these antimicrobial polymers insert in the membrane and rip it open. Hedrick adds that these antimicrobial polymers are biocompatible and biodegradable.

Hedrick and Yang hope to aim these nano-darts at methicillin-resistant *Staphylococcus aureus* (MRSA) and multidrug-resistant tuberculosis. “A bloodstream infection, [continued](#)»

Nanotechnology

MRSA, requires an injection,” says Yang, “but for TB, we are looking for alternative delivery strategies.” Since their approach provides so much control over the size and properties of the antimicrobial polymers, they can experiment to see what parameters provide the best therapies for different applications. For example, Hedrick mentions that they recently created a nano-size antimicrobial polymer that knocks out gram positive and negative bacteria—such as MRSA and *E. coli*, respectively—as well as yeast and fungi.

COMBINING PARTICLES AGAINST CANCER

Part of the challenge of using nanoparticles for drug delivery starts with combining the pieces. That turns out to be easy for James Tour, T.T. and W.F. Chao Chair in Chemistry at **Rice University** in Houston, Texas. He uses carbon clusters that are only a couple nanometers wide and 40–60 nm long. To make them soluble, he covers the surface with polyethylene glycol. Then, he puts the so-called functionalized carbon clusters in a solution that contains a tumor-targeting monoclonal antibody called cetuximab and a chemotherapeutic called paclitaxel. “Just shake it up, and they find their place,” Tour says. That is, the three pieces bind together during the shaking, but they don’t make hard-to-break covalent bonds. “That way, you don’t have to hope that an enzyme comes along later to cleave off the drug,” Tour says.

Originally, Tour developed this therapy for head and neck cancers. Using this trio of carbon clusters, antibodies, and drug with radiation proves very effective. In fact, the two therapies combine to give more than the sum of the parts. “The radiation causes the cancer cells to express more [epidermal growth factor] receptors on their surface and the antibody binds to those sites,” Tour explains. “More targets leads to more drug delivered to the tumor.”

However, Tour doubts that this approach will move forward on the regulatory pathway for head and neck cancers because effective treatments already exist. Instead, he might aim at diseases that still need better therapies, such as pancreatic cancer and glioblastoma multiforme in the brain.

Other investigators also study nanoparticle-based drug delivery. For example, nanotechnology could help researchers target the limited number of surface receptors on tumor cells. Only some receptors make good targets, but they do not cover the entire surface of every tumor cell. Moreover, receptors continually turn over, with old ones being broken down and new ones being constructed, which further limits the availability of such receptors as sites for drug attacks. That made Erkki Ruoslahti, distinguished professor at **Sanford-Burnham Medical Research Institute** in La Jolla, California, interested in combining tumor-targeting peptides with drug-bearing nanoparticles.

“It seems like a perfect marriage,” Ruoslahti says. “Peptides have a relatively low affinity for their targets, but you could put many peptides on the surface of a particle, which makes up for their low affinity. Plus, you can add a payload.”

Other approaches for treating cancer rely on drugs migrating to the desired site via leaky blood vessels in tumors. “This is very inefficient,” Ruoslahti says. “Plus, nanoparticles are not very good at getting out of blood vessels.” So

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Ruoslahti’s team is working with tumor-penetrating peptides. These peptides target blood vessels around tumors and bind to the surface protein neuropilin-1, which activates a transport pathway that carries the proteins from the blood vessel into the tumor. So, if Ruoslahti turns a nanoparticle into a drug carrier and coats it with these proteins, it will find a tumor, work its way into it, and bring the drug right where it needs to be. “It could get the nanoparticles deep into the tumor tissue,” Ruoslahti says.

So far, some of the tumor-penetrating peptides seem specific to certain kinds of tumors and some seem to be more general-purpose. Ruoslahti adds that the tumor needs to express neuropilin-1. “Most do, and even overexpress it,” he says, “but some probably don’t.” His academic group is already moving one protein, called iRGD, to the clinic. “This protein includes an integrin-binding sequence that I discovered almost 30 years ago,” Ruoslahti says, “and we can make it a tumor-penetrating peptide.”

This approach could go far beyond cancer. Ruoslahti points out that the target could be atherosclerotic plaque, inflammatory arthritis, and so on. “Pretty much any disease is targetable,” Ruoslahti says. “It will be a very general approach.”

Still, Ruoslahti points out one fundamental issue that must be handled to make nanoparticles effective at delivering drugs. “Nonspecific uptake by the liver and spleen have not really been solved,” he says. “That reduces the ability of specific targeting and causes the potential for liver toxicity.” He adds, “It would be better if the nanoparticles fall apart and don’t concentrate except at the target.” Making that happen, though, will take more research.

Mike May is a publishing consultant for science and technology.

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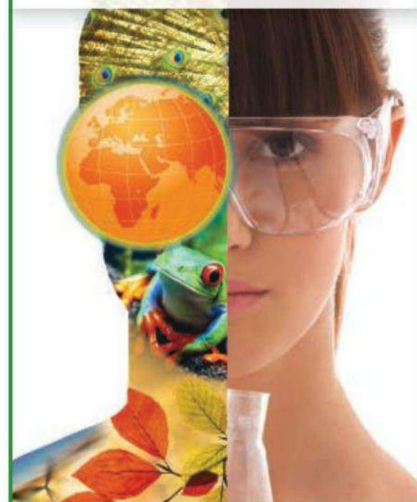
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The required documents differ according to the laboratory. Refer to the each URL mentioned above for more details.

[Deadline]

5:00 p.m. on Friday, September 28, 2012 (Japan Standard Time)

[Start of Employment]

April 1, 2013 or later, but negotiable

[Submitting Documents and Making Inquiries] Research Affairs Section, Advanced Research Promotion Division, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198 Japan
E-mail: rps-saiyo24 (please add "@riken.jp" to complete the address) Email attachments and telephone calls cannot be accepted. When you mail your application, please send as certified mail so that there will be a record of delivery. Please write in red on the front of the envelope, the name of the laboratory you are applying for.

<http://www.riken.jp/engn/r-world/info/recruit/index.html>



KECK GRADUATE INSTITUTE
of Applied Life Sciences

Junior Faculty Positions in Applied Life Sciences

Keck Graduate Institute is seeking applicants for several positions in applied life sciences to educate graduate students for careers in life sciences industries and to develop research programs that contribute new knowledge that will impact human health. We seek collaborative and entrepreneurial individuals with expertise in pharmaceutical discovery and development, biochemical engineering and bioprocessing, genomics and proteomics of personalized medicine, population genetics, and biomedical diagnostics and devices. A Ph.D. and postdoctoral experience in life science or engineering is required.

As part of recent strategic planning, KGI is focusing on rare and intractable disease as the theme for this faculty recruitment effort. We seek to build upon our successes in biomarker research, rare disease therapy, microfluidics, nucleic acid diagnostics, and systems biology to develop collaborations that will impact the ongoing discovery and development of diagnostics and therapies that address stratified medicine. We expect collaborations between biologists, chemists, and engineers to perform interdisciplinary research and development of technologies and therapies.

KGI offers unique programs to prepare its graduates for jobs in life science industry, government, and non-profits. These include a professional science masters (PSM), a postdoctoral professional masters (PPM), as well as MS and Ph.D. degrees in Applied Life Sciences. KGI is also launching a new School of BioPharmacy that may stimulate opportunities for joint appointments in both Applied Life Sciences and BioPharmacy.

Applicants should electronically submit a curriculum vitae, a synopsis of professional goals and research interests, and arrange for at least three letters of recommendation to www.kgi.edu.



UNIVERSITY of
ROCHESTER
MEDICAL CENTER

Faculty Position in Respiratory Toxicology

The University of Rochester Department of Environmental Medicine is expanding its Lung Biology Program seeking applications for a tenure-track faculty position at the **Assistant or Associate Professor** level. Candidates applying cutting-edge approaches to respiratory toxicology and lung biology to areas such as mechanisms underlying effects of particulate – including nanoparticles – and gaseous airborne contaminants on the respiratory, cardiovascular and central nervous system are of particular interest. The successful candidate would complement programs in respiratory immunology, infection, lung development, life span effects, stem cells and cognitive consequences of toxicants. Establishing an independent research program, a strong commitment to excellence in research, scholarship and teaching and a willingness to work within a cross-disciplinary team are essential.

The Department of Environmental Medicine has a long-standing NIEHS Environmental Health Sciences Center of Excellence with a well-equipped 3500 sq. ft. state-of-the-art barrier-type Inhalation Facility for human and animal exposures to laboratory-generated and ambient air contaminants. In addition, the Department is the administrative home to an NIEHS Toxicology Graduate Training Program. The University of Rochester recently was ranked as one of the top 15 Best Places to Work in Academia (*The Scientist*, July 2010) and includes a Clinical and Translational Sciences Institute and growth areas of neuromedicine, cancer, cardiovascular disease, immunology and infectious disease and musculoskeletal disease. Competitive salary and start-up packages are provided.

Requirements include a Ph.D. or M.D. degree with significant postdoctoral experience. Applicants should send a CV, a statement of research interests including future plans, and the names of 3-5 references to: **Dr. Deborah A. Cory-Slechta, Search Committee Co-Chair, Box EHSC, Department of Environmental Medicine, University of Rochester School of Medicine, Rochester, NY 14642** (deborah_cory-slechta@urmc.rochester.edu).

The University of Rochester is an Equal Opportunity Employer.



Aalto University

Finland is one of the top performing countries in the world in terms of education, innovation and the quality of life. A flagship project of the recent university reform in Finland, Aalto University was established 2010 by a merger of three Finnish universities: Helsinki School of Economics, Helsinki University of Technology and the University of Art and Design Helsinki. Our goal is to become a world class university by the year 2020, inspiring innovation and entrepreneurship by combining art and science with technology and business (www.aalto.fi/en/about/strategy).

Aalto University is now seeking to appoint three

Deans

to lead the operations of the

School of **Chemical Technology** (chem.aalto.fi/en),

School of **Engineering** (eng.aalto.fi/en) and

School of **Science** (sci.aalto.fi/en), respectively.

The Deans report directly to the University President and are members of the senior leadership team of the University. The Dean has overall responsibility for directing the school to achieve our world-class targets. He/she will be leading the development and implementation of the school's strategy and is responsible for day-to-day management of the school within the framework of the Aalto University strategy and policies. The Dean has a central role in setting school-level priorities for research and education as well as deciding on the allocation of resources for the academic departments. A key task for the Dean is the recruitment of new faculty according to the recently established Tenure Track career system.

A successful candidate will have

- a doctoral degree and proven track record of research and education excellence
- strong, international academic background, and experience in multidisciplinary research
- outstanding strategic capability to drive change by promoting creative solutions and by inspiring people
- ability to develop, implement and promote institutional objectives
- experience of leading academic communities, and evaluating and mentoring faculty
- excellent interpersonal skills to support effective communication between all stakeholders
- fluency in English, ability to speak Finnish or Swedish is an advantage

The Deans are appointed by the Board of the University for a five-year term. Salary is negotiable depending on qualifications and experience. Aalto University is an equal opportunity employer, particularly striving to achieve a more equal gender balance in our university.

Further information:

Ilkka Niemelä, Deputy President,
Tel. +358 50 511 3013, e-mail ilkka.niemela@aalto.fi

How to Apply:

Complete application package should include the following:

- Application Letter & Resume / CV
- Copy of transcript supporting the highest earned degree
- At least 3 professional letters of reference

Completed application package should be preferably e-mailed to registry@aalto.fi in pdf format. It is also possible to mail application to postal address Aalto University Otaniemi Registry Office, PO Box 11000, 00076 AALTO, Finland.

**Applications should be submitted by
Friday, September 28, 2012.**



SAHA INSTITUTE OF NUCLEAR PHYSICS
KOLKATA, INDIA
www.saha.ac.in

Faculty Positions

SINP invites applications for faculty positions at different levels from scientists having outstanding academic records and research achievements. At present, major research activities of the Institute are in the following broad areas: Atomic, Nuclear and Plasma Physics, Condensed Matter & Surface Physics, High Energy Physics, Theoretical & Mathematical Physics and Biophysical Sciences. SINP is an autonomous organization funded by the Department of Atomic Energy of Government of India to carry out basic research in the above broad areas. SINP has excellent state-of-the-art in-house facilities and also has collaborative access to various high-energy accelerators and synchrotron facilities across the world. We seek applicants, who will complement and extend our current research activities. Prospective applicants should also have interest in graduate teaching. This is a rolling advertisement (refer http://www.saha.ac.in/cs/www/position/Selection_Procedure.pdf) and applications may be submitted anytime of the year in plain papers/e-mails with details like cover letter, resume giving entire list of publications, a statement of research plan and contact details of 5 referees. Salary and rank will be commensurate with qualifications and research experience - minimum of two years of postdoctoral experience is required.

Director, Saha Institute of Nuclear Physics,
1/AF, Bidhannagar, Kolkata 700064, INDIA;
director.sinp@saha.ac.in



**UNIVERSITY OF
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The Professorship of Genetics

The Board of Electors to the Professorship of Genetics invite applications for this Professorship from persons whose work falls within the broadly defined field of Genetics, to take up appointment on 1 October 2012 or as soon as possible thereafter. The successful candidate would normally be expected to become the next Head of the Department for a minimum period of five years.

Further information is available at:

www.admin.cam.ac.uk/offices/academic/secretary/professorships/ or contact the Academic Secretary, University Offices, The Old Schools, Cambridge, CB2 1TT, (email: ibise@admin.cam.ac.uk), to whom a letter of application should be sent, together with details of current and future research plans, a curriculum vitae, a publications list and form CHRIS/6 (parts 1 and 3 only) with details of two referees, so as to reach him no later than 6 August 2012.

Informal enquiries may be made to Dr. Cahir O'Kane, Head of the Department of Genetics, in the first instance by email (head@gen.cam.ac.uk)

The University is committed to Equality of Opportunity.



TEXAS A&M
HEALTH SCIENCE CENTER
COLLEGE OF MEDICINE
INSTITUTE FOR REGENERATIVE MEDICINE

Post-doctoral Positions

The Texas A & M Institute of Regenerative Medicine is seeking Ph.D. level post-doctoral fellows for research on adult stem/progenitor cells referred to as mesenchymal stem cells or multipotent mesenchymal cells (MSCs). The Institute is dedicated to research both on the basic biology of MSCs and the therapeutic products they produce. Current research includes development of new therapies for diseases of the eye, myocardial infarction, cancer, diabetes, stroke, epilepsy and traumatic brain injury. The Institute occupies newly renovated laboratories and a series of core laboratories equipped with state-of-the-art instrumentation. It also includes a newly renovated vivarium for small animal experiments. Post-doctoral appointments will be for one year with the opportunity to renew for a second and third year subject to performance. Salaries and benefits are competitive. Candidates should have excellent verbal skills and a Ph.D. or M.D. degree from a well recognized university.

Before August 15, 2012, please visit the website at <http://www.tamhsc.edu/> and complete the application for posting # 2012309 by **August 15, 2012**. The application should include curriculum vitae, brief statement of research interests, and three letters of recommendation.

The Texas A&M Health Science Center is an AA/EO Employer.

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Alexis and Noah Beery

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Contact the Admissions Office, telephone: 800-824-5526 at the New England College of Optometry, 424 Beacon Street, Boston, MA 02115. Additional information at website: <http://www.neco.edu>, e-mail: admissions@neco.edu.

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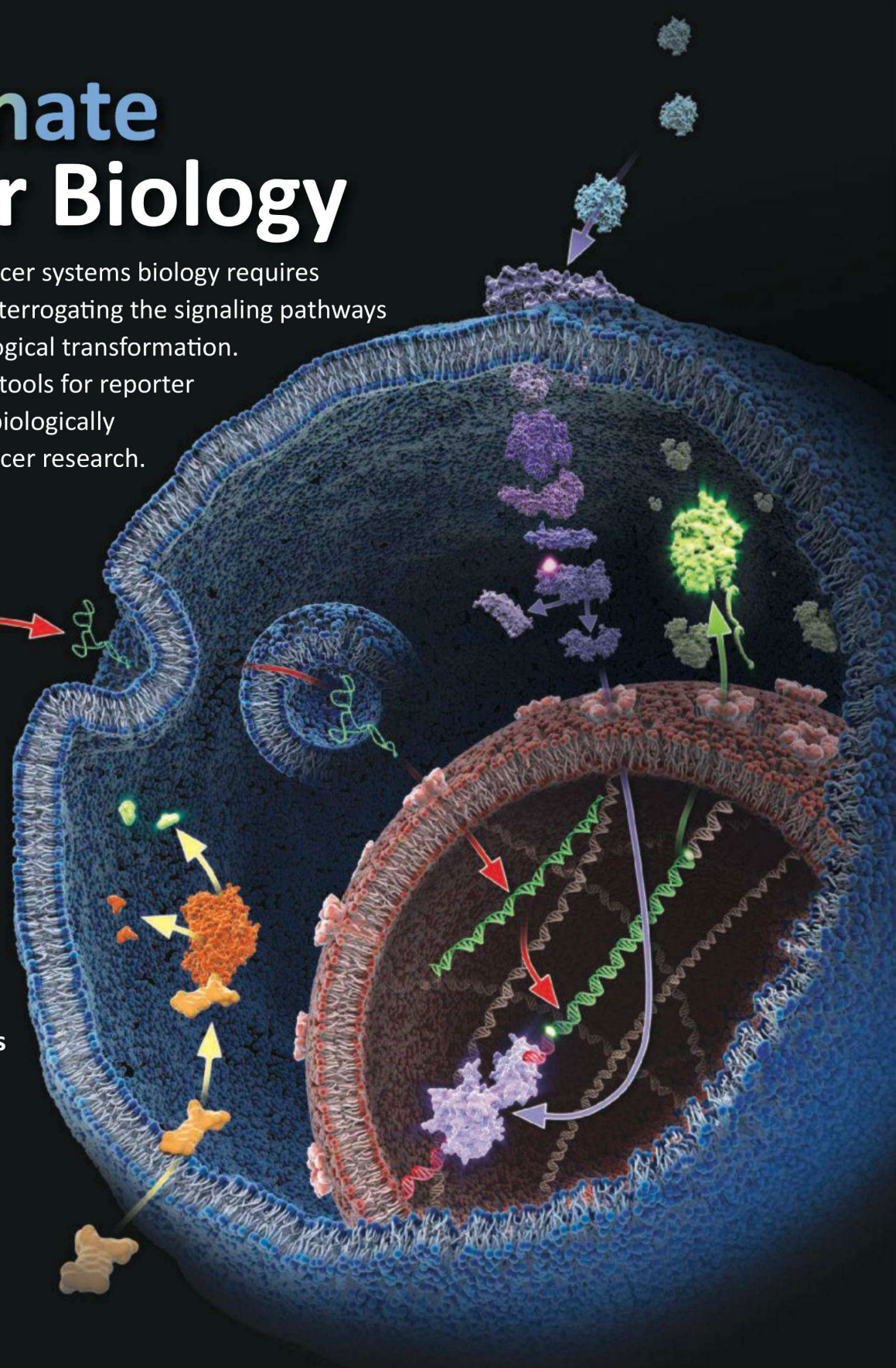
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